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INFLUENCE OF THE FLUORINE ION ON THE GROWTH *IN VITRO* OF HUMAN AMNION CELLS, T (KIDNEY) CELLS, AND HeLa CELLS

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CONTENTS

1 INTRODUCTION

Though it appears to be agreed that the fluorine ion depresses the growth *in vitro* of human and murine cells opinions are still divided as regards the concentration at which this effect makes itself felt

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BERRY and TRILLWOOD (1963) report that addition of as little as 0 1 mg/l of sodium fluoride (about 0 045 ppm fluorine ion) to the culture medium is sufficient markedly to depress the growth *in vitro* both of human HeLa cells and of mouse fibroblasts, as determined by counting the cells with the aid of a Coulter counter after trypsinization From additional clonal culture experiments they conclude that since much higher concentrations are necessary to cause a significant decrease in reproductive capacity, the growth reduction is probably due to a decrease in division rate As is apparent from their table, growth depression after addition of 0 45 ppm fluoride ion would seem not to differ appreciably from that after addition of 0 045 ppm

ARMSTRONG and collaborators (1965a) repeated and extended these experiments and observed results contrary to those of Berry and Trillwood Working with two established lines of human epithelial cells *viz* HeLa cells and oesophageal epithelium cells and using besides cell counts determinations of cell protein as an index of cell multiplication, they found that concentrations up to 10 ppm of fluorine ion are without effect An incipient retardant effect was observed at a concentration of 15 ppm They refer to experiments by PROFFIT and ACKERMANN (1964) who found that the uptake of tritium labelled thymidine by organ cultures of rat metacarpal bones was not affected by up to 20 ppm, indicating unimpaired DNA formation, and to work by ALBRIGHT (1964) who found no inhibition of multiplication of murine leukaemic lymphoblasts at 5 7 ppm

After BERRY and TRILLWOOD (1965) had remarked that the growth rate in Armstrong s HeLa cell experiments was very low to begin with [4]) ARMSTRONG c s (1965b) came up with new experiments where the growth rate was comparable to that observed by Berry and Trillwood

In these experiments growth was measured in a different way after 24 h of incubation the position in each of the flasks of 5 cells which had divided once was marked The number of cells in each of these clones was recorded but clones which had not divided into 4 cells on day 2 were

[4]) Their remark that the growth rate of Armstrong s oesophageal epithelial cells was much less than that of their own mouse fibroblasts and HeLa cells is not germane to the issue, it is well known that there are marked differences in growth rate between cell types

abandoned Obviously, this procedure results in an increase of the measured growth rate The discrepancy between the earlier results of the Armstrong group and those of Berry and Trillwood remains unexplained however No statement is made as to whether or not at the higher fluoride concentrations more clones had to be abandoned because of a standstill in growth

The graph presented shows a surprisingly regular exponential growth, at exactly the same rate, with and without addition of 10 ppm fluorine ion, no dispersions are, however, indicated Finally NIAS (1965), a former collaborator of Berry and Trillwood, presents the results of a small series of experiments in which he finds no effect, on the growth of HeLa cells, of 1 ppm NaF

The present paper is a report on experiments where the influence of fluoride ion, added in concentrations of 1, 2, 4, 8 and 16 ppm to the culture medium, on the growth of human HeLa cells, amnion cells and T (kidney) cells was studied

2 EXPERIMENTAL

2 1 METHODS

The cells were grown in 250 ml closed culture flasks (Kimax) at 37 °C The culture medium consisted of 5 per cent calf serum and 95 per cent Hank's balanced salt buffer solution, with 5 mg/ml lactalbumin, 0 5 mg/ml streptomycin, and 1000 U/ml penicillin added Each flask contained 12 ml of medium and 1 ml of inoculate with, initially about 500 000 cells The medium was changed every 48 h

Sodium fluoride was added to obtain fluorine ion concentrations of either 1 2 4 8 or 16 ppm over any already present in the medium [5])

As preliminary experiments had shown that the results of DNA determinations were in concord with those of cell counting but that their dispersion was markedly less, growth was measured by determination of the DNA content of the culture flasks after 2, 3 4, 5 6 and 7 days of cultivation

The medium is removed and the cell layer is washed once with saline The cells are then loosened from the glass surface with

[5]) The fluoride content of Leiden drinking water is exceptionally low.

5 ml 0 25 per cent trypsin in saline and, together with 5 ml saline, transferred to an 18 ml closed culture tube (Kimax) and centrifuged

After careful removal of the supernatant the tubes are stored at 4 °C DNA determinations are made according to CERIOTTI's (1952) indole method after extraction of the cell residues with 1 ml of 1 M perchloric acid

For each fluoride solution, two cultures were grown, in separate flasks, for each period of growth Hence for each NaF concentration, and each day, the two DNA determinations, whether made in duplicate or not, provide independent estimates of the corresponding amount of growth

T cells and amnionic cells were analysed in duplicate and HeLa cells were analysed singly against standard solutions of purified thymus DNA The reaction mixture consisted of 0 25 ml of per chloric acid extract and 3 75 ml reagent (26 mg indole and 66 ml concentrated hydrochloric acid in 250 ml)

To determine the amount of DNA *initially present in the flasks*, 1 ml inoculate samples, containing some 500 000 cells, of each of the cell strains were analysed The results of these analyses are shown in Table 1

TABLE 1

		DNA											
T- *Cells*	duplicate *analysis*	18 22	8 14	6 3	17 18								average 13
Amnion Cells	duplicate analysis	2 6	10 10	5 5	3 4	4 4	3 10	9 9	3 5	5 6	3 4		average 6
HeLa *Cells*	single *analysis*	5	5	15	21	7	7	14	9	10	4	1	average 9

2 2 RESULTS

Raw data are given in Tables 2 to 4, which show the amount of DNA in μg per culture flask, after 2, 3, 4, 5, 6 and 7 days of cultivation, for 0, 1, 2, 4, 8 and 16 ppm F^+ added to the culture medium The form of the growth curves can be seen by reading down the columns Growth is slow at first and becomes much more rapid irregularly

T Cells DNA values in µg per flask

Days of cultiv	ppm F^+	0 *Dupl* Culture	1 Dupl Culture	2 Dupl Culture	4 Dupl Culture	8 Dupl Culture	16 Dupl Culture
0	Dupl DNA Determ	18 8 20 11 22 14	6 17 4 18 3 18				
2	Dupl (Trpl) DNA Determ	11 14 12 18 13 22	9 9 12 9 11 9 13	21 17 31 27 21 30 25	21 14 22 14 22 14	11 5 10 8 9 11	11 30 14 32 17 34
3	Dupl DNA Determ	19 14 20 14 21 14	26 25 24 24 22 22	14 32 13 29 12 26	11 15 12 16 12 16	10 16 18 18 19 19	23 15 23 16 23 18
4	Dupl (Trpl) DNA Determ	41 30 42 31 43 32	11 14 14 13 14 14 14	28 17 29 19 32 21	30 25 28 26 26 28	27 29 28 28 30 31	19 23 22 24 26 24
5	Dupl DNA Determ	52 60 54 64 55 68	60 11 63 12 66 13	69 47 67 42 65 44	50 39 53 38 56 38	24 35 24 36 24 37	22 27 24 29 27 31
6	Dupl DNA Determ	57 119 60 122 62 125	87 99 87 102 87 106	83 96 85 102 87 109	74 83 76 86 78 88	60 60 62 62 64 64	56 98 61 100 66 102
7	Dupl DNA Determ	102 138 104 140 106 142	151 133 147 136 143 138	151 147 147 150 143 152	139 107 140 105 141 103	134 68 130 74 127 80	94 88 94 88 95 89

TABLE 3

Amnion Cells DNA values in μg per flask

Days of cultiv	ppm F+	0 Dupl Culture	1 Dupl Culture	2 Dupl Culture	4 Dupl Culture	8 Dupl Culture	16 Dupl Culture
0	Dupl	2 10	5 3	4 3	9 3	5 3	
	DNA	4 10	5 4	4 6	9 4	6 4	
	Determ	6 10	5 4	4 10	9 5	6 4	
2	Dupl	11 12	— 3	11 10	6 12	17 15	7 10
	DNA	13 12	4	12 10	7 12	18 15	6 10
	Determ	15 11	— 5	12 10	8 12	20 15	5 11
3	Dupl	11 13	19 19	16 24	21 17	23 22	12 15
	DNA	14 24	21 20	18 24	22 17	24 23	12 16
	Determ	16 25	23 21	20 25	24 17	24 24	12 16
4	Dupl	47 30	44 17	34 39	47 19	50 31	29 41
	DNA	48 33	45 18	36 40	47 19	52 30	30 39
	Determ	50 36	46 18	37 41	47 19	53 29	31 37
5	Dupl	88 85	76 78	67 70	69 93	91 78	77 77
	DNA	88 84	77 78	68 70	70 88	93 76	76 80
	Determ	88 84	78 77	69 71	72 84	95 75	74 85
6	Dupl	109 133	118 138	133 109	156 133	134 123	103 114
	DNA	108 134	118 140	134 111	155 134	134 123	104 111
	Determ	107 135	117 142	135 113	154 134	134 123	104 108
7	Dupl	187 156	197 189	201 191	192 176	94 183	96 135
	DNA	188 159	197 190	202 192	196 178	95 183	96 135
	Determ	189 162	197 190	202 194	200 180	96 183	96 135

TABLE 4

HeLa Cells DNA values in μg per flask

…m F+ of v	0		1		2		4		8		16	
	Dupl	Cult	Dupl	Cult	Dupl	Cult	Dupl	Cult	Dupl	Cult	Dupl	Cult
	5	5	15	24	7	7	11	9	10	6	7	—
	14	10	14	14	7	20	20	19	4	13	18	17
	33	24	22	21	20	14	21	21	17	25	23	28
	42	29	42	57	27	50	28	38	25	26	42	45
	42	51	48	54	42	45	56	61	31	28	37	47
	102	—	61	105	67	56	42	39	42	—	57	32
	88	94	119	92	56	111	93	82	68	101	74	80

The differences between the duplicate determinations remain small throughout except for some of the small amounts of DNA, but the variability between the replicate determinations increases rapidly as we read down the columns and this trend seems to be very irregular

3 STATISTICAL ANALYSIS

3 1 A SUITABLE SCALE FOR DETAILED ANALYSIS

In contrast to the case for many other biological dose response relationships there seem to be as yet no standardized procedures of statistical analysis when the response is a variation in the rate of growth and division of cells Also the mathematical forms of growth curves of this kind are not known We have therefore estimated the form of these curves empirically, but we have not tried to fit a mathematical function to this form

Both biologically and statistically it is reasonable to analyze growth curves in terms of the logarithm of the amount of the growing substance The relative variation whether random or systematic is more important biologically than the absolute variation, and it is clear that the random scatter is more nearly homogeneous in a logarithmic scale This applies both to the pair variances and the variation along the rows

In terms of log y, where y is in μg DNA, variances for large y were fairly homogeneous, but not those for small y, the pair variances for 2 days were too large, and those for 0 days (initial

TABLE 3

Amnion Cells DNA values in μg per flask

Days of cultiv	ppm F+	0 Dupl Culture	1 Dupl Culture	2 Dupl Culture	4 Dupl Culture	8 Dupl Culture	16 Dupl Culture
0	Dupl	2 10	5 3	4 3	9 3	5 3	
	DNA	4 10	5 4	4 6	9 4	6 4	
	Determ	6 10	5 4	4 10	9 5	6 4	
2	Dupl	11 12	— 3	11 10	6 12	17 15	7 10
	DNA	13 12	4	12 10	7 12	18 15	6 10
	Determ	15 11	— 5	12 10	8 12	20 15	5 11
3	Dupl	11 13	19 19	16 24	21 17	23 22	12 15
	DNA	14 24	21 20	18 24	22 17	24 23	12 16
	Determ	16 25	23 21	20 25	24 17	24 24	12 16
4	Dupl	47 30	44 17	34 39	47 19	50 31	29 41
	DNA	48 33	45 18	36 40	47 19	52 30	30 39
	Determ	50 36	46 18	37 41	47 19	53 29	31 37
5	Dupl	88 85	76 78	67 70	69 93	91 78	77 77
	DNA	88 84	77 78	68 70	70 88	93 76	76 80
	Determ	88 84	78 77	69 71	72 84	95 75	74 85
6	Dupl	109 133	118 138	133 109	156 133	134 123	103 114
	DNA	108 134	118 140	134 111	155 134	134 123	104 111
	Determ	107 135	117 142	135 113	154 134	134 123	104 108
7	Dupl	187 156	197 189	201 191	192 176	94 183	96 135
	DNA	188 159	197 190	202 192	196 178	95 183	96 135
	Determ	189 162	197 190	202 194	200 180	96 183	96 135

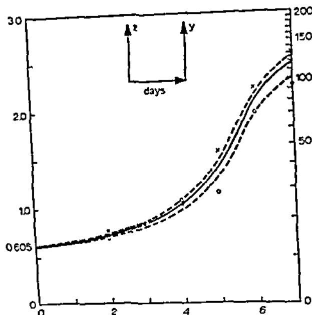


Fig 1a T-cells

Standard errors of plotted points in z scale
×, 032, ○, 045, difference 035 (For day 5 these three estimates should *be doubled*)

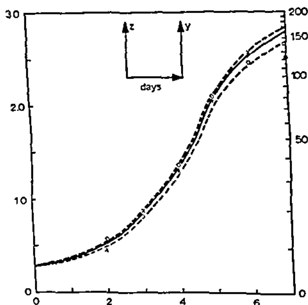


Fig 1b Amnion cells

Standard errors of plotted points in z scale
×, 023, ○, 033 difference 041 (For day 4, 058, 080 and 095 respectively).

amounts of DNA), larger still. The growth curves did not become linear, in other words, they *are not simple exponentials*.

A transformation was therefore chosen that is linear for the smaller values of y and approaches $\log y$ for larger ones. We have put

$$z = \sinh^{-1}(y/20) \qquad (1)$$

$$= y/20 - 1/6\,(y/20)^3 + \ldots \qquad (2)$$

so that

$$y = 10\, e^{z}(1 - e^{-2z}) \qquad (3)$$

Hence, when y is less than about 15, $z = y/20$, and when $\exp -2z = 1$, $z = \log_e(y/10)$. The variances were more nearly homogeneous in this scale, which was therefore used for all the statistical analyses. The resulting estimates for different fluoride concentrations were then converted back to the original (y) scale, and expressed as ratios of a standardized growth curve as described below.

Table 5 gives the means and variances in the transformed (z) scale, and the means of the z's transformed back to the y scale. In relation to the duplicate determinations from the same flask, the analysis is in terms of the inverse hyperbolic sine of the average of the two y values. Hence there are two z values for each day and each fluoride concentration obtained from separate independently growing cultures, making 12 for each day.

From the means of these 12 values average growth curves were obtained for each type of cell.

TABLE 5

The means over all doses and variances estimated from pair differences, in the transformed scale

$z = \sinh^{-1} y/20$ where y is in μg DNA

z = mean over all doses for the same number of days

y = the same transformed back to μg DNA, thus $y = 20 \sinh z$

Days	T Cells			Amnion Cells			HeLa Cells		
	z	var z	y	z	var z	y	z	var z	y
0	0.605	0.0870	12.8	0.289	0.0129	5.8	0.445	0.0498	9.2
2	0.729	0.0462	15.9	0.510	0.0176	10.6	0.641	0.0409	13.7
3	0.828	0.0372	18.5	0.858	0.0218	19.3	0.957	0.0188	22.2
4	1.038	0.0151	24.7	1.328	0.1278	35.1	1.366	0.0454	36.6
5	1.426	0.1742	39.2	2.079	0.0075	78.7	1.536	0.0091	44.3
6	*2.115*	*0.0661*	*81.7*	*2.528*	*0.0116*	*124.4*	1.751	0.0692	*55.9*
7	2.478	0.0397	118.3	2.702	0.0484	162.5	2.170	0.0554	86.4

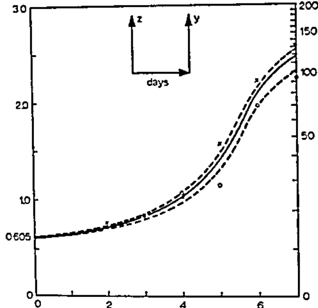


Fig 1a T-cells
Standard errors of plotted points in *z* scale
×, 032, ○, 045, difference 055 (For day 5 these three estimates should be doubled)

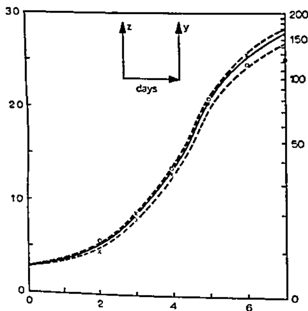


Fig 1b Amnion cells
Standard errors of plotted points in *z* scale
×, 023, ○, 033, difference 041 (For day 4, 058, 080 and 095 respectively).

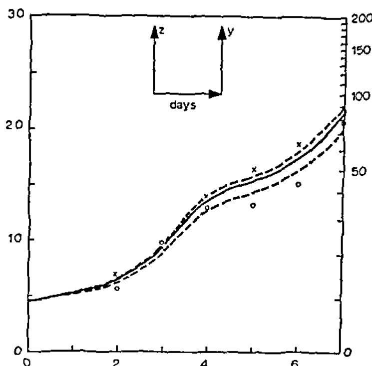


Fig 1c HeLa cells

Standard errors of plotted points in z scale
×, 029, ○, 042, difference 051

Fig 1 Growth curves for the three cell types, showing the two scales for measuring growth y scale, μg DNA per flask, z scale, $z = \sinh^{-1} y/20$
Full curve mean growth curves for all fluoride concentrations
Top curve estimated *growth curve for* concentrations 0, 1, 2 and 4 *ppm*
Bottom curve estimated growth curve for concentrations 8 and 16 ppm

×, observed means, 0, 1, 2 and 4 ppm
○, ,, ,, 8 and 16 ppm

All means were obtained in the z scale

3 2 THE AVERAGE GROWTH CURVE

We first consider the mean (in the transformed scale) for all the determinations made on one day These means are plotted against the length of the growth period in the middle curves in figs 1 (a–c) Both the transformed scale and the original DNA scale are given The top and bottom curves are estimated growth curves for the means for doses 0 1 2 and 4 and for doses of 8 and 16 (The method of obtaining them is discussed later) The points plotted are the actual means for these two dose groups

The shapes of the growth curves are clearly different for the three cell types For T cells there is a rapid increase (even in the transformed scale) between 5 and 6 days from the beginning of growth and there is a similar increase for amnion cells between 4 and 5 days For HeLa cells also there are variations in the growth rate but this is less rapid and the averaged growth curve is more nearly linear between 2 and 7 days The pair variances are discrepantly high at 5 days for T cells and at 4 days for amnion cells that is just when the very rapid growth is beginning If this period of rapid growth should begin at different times in different individual growth curves this would explain why these particular variances are so much higher than the others For HeLa cells with a less variable average growth rate the pair variances seem to be reasonably homogeneous throughout

Table 6 gives all the pair means expressed as deviations from the corresponding row means in other words the entries are stan

TABLE 6

The deviations in the pair means from the means for all doses for the same day

(a) T cells

Days	Concentrations						Row means
	0	1	2	4	8	16	
2	— 037	— 239	+ 291	+ 061	— 294	+ 221	720
3	— 063	+ 182	+ 062	— 198	— 018	+ 037	828
4	+ 317	— 408	— 028	+ 077	+ 097	— 053	1 038
5	+ 369	— 211	+ 279	+ 129	— 240	— 376	1 426
6	+ 045	+ 145	+ 125	— 005	— 265	— 045	2 115
7	+ 017	+ 172	+ 222	+ 092	— 183	— 253	2 478
	means (without day 5)						
	+ 005	— 030	+ 134	— 009	— 133	— 019	1 438

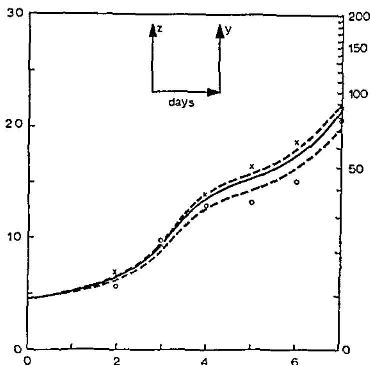


Fig 1c HeLa cells

Standard errors of plotted points in z scale
×, 029, ○, 042 difference 051

Fig 1 Growth curves for the three cell types, showing the two scales for measuring growth y scale, μg DNA per flask, z scale, $z = \sinh^{-1} \sqrt{y/20}$
Full curve mean growth curves for all fluoride concentrations
Top curve estimated growth curve for concentrations 0, 1, 2 and 4 ppm
Bottom curve estimated growth curve for concentrations 8 and 16 ppm

×, observed means, 0, 1, 2 and 4 ppm
○, ,, ,, 8 and 16 ppm

All means were obtained in the z scale

3 3 ANALYSIS OF THE DEVIATIONS FROM THE AVERAGE GROWTH CURVE

Table 7 gives the analysis of variance for the deviations given in Table 6, again leaving out the two rows with much larger variances The significance levels given only refer, of course, to the amount of variation between the column means, disregarding any trend in this variation

TABLE 7

Analysis of variance with $z = \sinh^{-1}(y/20)$ Sums of squares of deviations, and variances for 100 z All deviations are from the row means The pairs term is obtained from the terms $\frac{1}{4}(z_1 - z_2)^2$, one for each pair The column term is due to the difference between the column means for the different dose (concentration) effects, and thus gives the main dose effect The doses × days term expresses the effect of the different doses on the shape of the growth curve, i e variations in the growth curve over and above those described by a parallel shift P is the probability of obtaining a greater column variance/pairs variance ratio by chance, if there is really no dose effect on the column means

	sum	df	variance
T-cells without day 5			
Pairs	12259 0	30	408 6
Columns	4001 0	5	800 2
Doses × days	14761 6	20	738 1
Total	31021 6	55	
P	0 113		
Amnion cells without day 4			
Pairs	6407 5	30	213 6
Columns	3520 5	5	704 1
Doses × days	6615 7	20	324 3
Total	16453 6	55	
P	0 015		
HeLa cells			
Pairs	14329 0	36	398 0
Columns	8662 3	5	1732 5
Doses × days	13633 2	25	545 3
Total	36624 5	66	
P	0 003		

TABLE 6 (*continued*)

(b) Amnion cells

Days	Concentrations						Row means
	0	1	2	4	8	16	
2	+ 070	— 160	+ 005	— 055	+ 250	— 110	510
3	— 033	+ 042	+ 062	+ 012	+ 132	— 218	858
4	+ 121	— 159	+ 061	— 109	+ 101	— 024	1 328
5	+ 091	— 019	— 124	+ 001	+ 066	— 014	2 079
6	— 033	+ 027	— 023	+ 142	+ 032	— 148	2 528
7	+ 063	+ 168	+ 188	+ 138	— 207	— 352	2 792
	means (without day 4)						
	+ 032	+ 012	+ 022	+ 048	+ 055	— 169	1 753

Standard errors 049 for concentration 1,
046 for the other concentrations

Only one determination for day 2, concentration 1, missing value of z assumed to be 0 50

(c) HeLa cells

Days	Concentrations						Row means
	0	1	2	4	8	16	
2	— 076	+ 009	— 031	+ 224	— 236	+ 109	641
3	+ *193*	— *022*	— *192*	— *037*	— *047*	+ *103*	957
4	— 036	+ 264	+ 014	— 096	— 301	+ 154	1 366
5	+ 044	+ 129	— 016	+ 254	— 356	— 056	1 536
6	+ 334	+ 344	+ 084	— 296	— 226	— 241	1 751
7	+ 050	+ 185	— 090	+ 010	— 040	— 115	2 170
	means						
	+ 086	+ 152	— 038	+ 011	— 200	— 007	1 402

Standard errors 060 for concentrations 0 and 8
058 for the other concentrations

Only one determination for day 6, concentrations 0 and 8 Missing z values assumed to be 1 84 and 1 56 respectively

The results for all three cell types suggest a small irregular decrease in growth rate especially for concentrations of 8 ppm but the variation for T cells could be a chance one

dardized on the number of days of growth In obtaining the column means, day 5 for T cells and day 4 for amnion cells were left out These means give estimates of the effects of the different fluoride concentrations The standard errors are estimated from the com bined pair variances

TABLE 8

Weighted mean dose effects expressed on the z scale as deviations from the daily means ($\bar{z}$) for all doses and weighted by the estimated amount of growth up to that day (lower weight factors for day 5, T-cells and day 4, amnion cells see text), also the corresponding mean z values for concentrations 0, 1, 2, and 4 and for 8 and 16 ppm and their standard errors

Values of $z-\bar{z}$

Fluoride concentration, ppm

Cell type	0	1	2	4	8	16
T	+ 063	+ 075	+ 160	+ 013	— 181	— 136
	Standard errors 081					
Amnion	+ 036	+ 054	+ 033	+ 086	— 024	— 185
	Standard errors 053					
HeLa	+ 108	+ 199	— 027	— 027	— 191	— 062
	Standard errors 065 (072 for 0 and 8 ppm)					

Values of z

	0, 1, 2 4	S F	8 16	S E	Difference	S F
T	2 556	0 040	2 319	0 057	0 237	0 070
Amnion	2 347	0 027	2 190	0 039	0 157	0 046
HeLa	1 729	0 033	1 539	0 049	0 190	0 059

(μg DNA per flask, the y scale) In these units, the estimated amounts of growth for each day (as given in Table 4 by averaging over all doses) are shown in Table 9 The values for day 7 in the mean growth curve are regarded as the standard ones, and the amounts of growth for each concentration have been expressed as a ratio of this standard The amounts of growth for the other days can be estimated by multiplying each of these ratios by the entries for the standard growth curve, assuming of course, that the amounts of growth remain in the same proportion for the same growth period and different concentrations This seems to be approximately true, at least for the longer periods of growth

The corresponding estimates for the growth of the means for 0 1 2 and 4 ppm and for 8 and 16 ppm are shown in the figure The decrease from one to the other is clearly about 15 per cent for amnion cells, and more than 20 per cent for T and for HeLa cells

The 'doses × days' variance is larger than the corresponding pair variance but not greatly so Hence the growth curves for the same kind of cell and different fluoride concentrations do not vary greatly in shape in fact starting [of course] from the same point they become approximately parallel in the transformed scale

3 4 AN ESTIMATE OF THE AVERAGE EFFECT OF THE FLUORIDE CONCENTRATIONS ALLOWING FOR THE FACT THAT THE GROWTH CURVES ARE CUMULATIVE

In assessing the effects of different fluoride concentrations no allowance has so far been made for the fact that growth curves are cumulative If we do so we obtain another estimate of the average effect of each concentration which may be more meaningful biologically

In principle the deviations reading down the columns of Table 6 are simply *weighted by the amount of growth up to that point* These amounts of growth are estimated by subtracting the means for day 0 from the means for each of the other days (as given in Table 5) (The derivation is given in the appendix)

In estimating the mean dose effect on the T cells the weight for day 5 was multiplied by 1/4 and for amnion cells the weight for day 4 was multiplied by 1/6 This allowed for the discrepantly large variances for these particular days

Table 8 gives the weighted means obtained in this way together with their standard errors The pattern of variation with increasing fluoride concentrations is similar to that obtained from the unweighted means but it is more regular In particular the increase between concentrations of 8 and 16 ppm for T cells and HeLa cells which is *against* the general trend becomes less In any case the conclusion is that concentrations between 4 and 8 ppm begin to have an effect on T cells concentrations of about 8 ppm on amnion cells whilst for HeLa cells as little as 2 ppm may have some effect in diminishing the growth rate

For all three cell types there is a marked drop in growth rate between concentrations of 4 and 8 ppm The means for 0 1 2 and 4 ppm and for 8 and 16 ppm are given in the table the differences for all three cell types are between 3 and 4 times their standard errors

These estimates have been converted back to the original units

estimates for day 2 are obtained from all the other days including of course day 2 If we take the 6th day as the standard estimates based on earlier days become forecasts but the principle is the same

Each estimate must be weighted by a factor inversely proportional to the variance in obtaining the contribution to the estimate for day 2 due to the z value for day 7 the deviation $z_2 - z_0$ is therefore first multiplied by g_2/g_7 and then by g_7^2/g_2^2 Then from whichever day we start with we arrive at the same weighted mean z_w given by

$$z_w - \frac{g_2(z_2 - z_2) + g_3(z_3 - z_3) + \quad g_7(z_7 - z_7)}{g_2 + g_3 + \quad g_7} \tag{4}$$

The summation is of course over z values for the same fluoride solution

The variance of this estimate is slightly greater than $\Sigma g_i^2 \operatorname{var} z/(\Sigma g_i)^2$ This allows only for the random variation in the z s A rough calculation showed that the effect of errors in the estimates of the g s is much smaller This has been neglected We have also ignored the errors in the estimates of z_0 which are not negligible These would affect the relative values of the g s but have little effect on the ones that have most weight namely g_7 and g_6

4 DISCUSSION

SINGER and ARMSTRONG (1960) found that for fluoride concentrations in the drinking water ranging from 0 15 to 2 25 ppm fluoride (about 0 07 to 1 ppm fluorine ion) plasma fluoride concentration is apart from transient increases after meals within the range 0 14 to 0 19 ppm (some 0 06 to 0 09 ppm fluorine ion) The lowest concentration at which according to BERRY and TRILLWOOD fluorine added to the medium markedly depresses the growth *in vitro* of HeLa cells (and of mouse fibroblasts) is below the lower end of the physiological plasma range NEIL JENKINS (1963) rightly remarks that if normal cells *in vivo* should have the same sensitivity to fluoride as the cancer cells studied *in vitro* by Berry and Trillwood fluoride at physiological concentrations might act as a weak inhibitor of cell growth He also points out that without addition of any fluoride the concentration in the medium would in all probability already be higher than the lowest concentration added.

The results obtained in the present experiments are at variance

TABLE 9

Accumulated amounts of growth for each day, and the effects on them of the different fluoride concentrations, measured in μg DNA and expressed as ratios of the amount for the mean growth curve at 7 days The amounts of growth for shorter periods can be estimated, assuming they are in the same proportion, by multiplying the entries in the first part of the table by these growth ratios

Days	0	2	3	4	5	6	7
	Amounts of growth in μg DNA (y scale)						
T	(12 8)	3 1	5 7	11 9	26 4	66 8	105 4
Amnion	(5 9)	4 8	13 5	29 2	72 9	118 6	140 9
HeLa	(9 2)	4 5	13 0	27 4	35 1	46 6	77 2

	Concentrations, ppm						
	0	1	2	4	8	16	0, 1, 2, 4	8, 16
	Growth ratios and standard errors							
T	1 071	1 079	1 176	1 013	0 832	0 871	1 083	0 851
S E	098	099	107	093	077	080	050	056
Amnion	1 038	1 050	1 035	1 094	0 974	0 823	1 056	0 896
S E	057	058	057	060	054	046	028	035
HeLa	1 131	1 252	0 970	0 969	0 800	0 931	1 075	0 863
S E	092	091	073	073	069	064	041	050

Note The standard errors are approximate since they assume that the y values for zero concentrations are exact Errors in these can give rise to small errors in the corresponding growth rates, all in the same direction, the comparison between the growth ratios is affected very slightly

APPENDIX

THE DERIVATION OF THE WEIGHTED AVERAGE ALLOWING FOR CUMULATIVE GROWTH

The amount of growth for any particular number of days must provide information on the amounts of growth during previous days (we recall that all determinations were made on separate cultures)

Subtracting the zero values in Table 5 from each of the other row means we obtain estimates of the mean amounts of growth $g_2, g_3\ g_4 \quad g_7$ up to the corresponding days The estimate for day 2 from the day 7 value is $z_7 g_2/g_7$ with variance (g_2^2/g_7^2) var z Similarly

culture flasks, determined according to Ceriotti's indole method, was used as index of growth

For each of the fluorine concentrations, and for each of the cultivation periods used, viz 2, 3 4, 5, 6, and 7 days, two cultures were grown in separate flasks DNA determinations were made in duplicate in the case of T cells and amnion cells

In a detailed statistical analysis the shapes of the growth curves and their variability were taken into account It was found that concentrations between 4 and 8 ppm have an incipient growth-depressing effect on T-cells, concentrations of about 8 ppm begin to have an effect on amnion cells, for HeLa cells as little as 2 ppm may already have some influence From estimates based mainly on the longer growth periods it appears that for *all three cell types the average growths at 8 and 16 ppm were 15–20* per cent lower than at the other concentrations

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with those of Berry and Trillwood The fluoride concentration (over that already present in the medium) at which an incipient effect on the growth of HeLa cells was found exceeds the lowest value at which a marked depression of growth was observed by Berry and Trillwood by a factor 45.

We are unable to account for this difference, it cannot be ascribed to the difference in method (DNA determinations *vs* cell counting) because, as remarked earlier, the results of cell counting and of DNA determinations agreed Nor does it seem likely that an initially high fluoride concentration in their medium could be invoked According to the present experiments there is an incipient growth retarding effect at 2 ppm fluorine ion The range between 1 and 2 ppm was not subdivided, but it can be concluded that the concentration at which an effect makes itself felt lies within this range Supposing the initial concentration in Berry and Trillwood's medium to be of this order of magnitude it seems most unlikely that addition of 0 045 ppm would tip the scale, and even more unlikely that, if such were the case, there would be virtually no difference between the effect of addition of 0 045 ppm and that of addition of ten times that amount

On the other hand, the fluorine concentration (2 ppm) where, in the present experiments, a depressing effect on HeLa cells begins to make itself felt, is five times less than the one (10 ppm) which was still without effect in the hands of Armstrong c s This difference might be due to a selection effect if it should indeed be true that in their experiments more clones had to be abandoned at the higher fluoride concentrations No explanation can be offered for the fact that, unlike Anderson c s, we found growth not to be exponential for any of the cell types

The fact that the cancer (HeLa) cells seem to be more sensitive to fluorine than the other human cell types studied in the present investigation is of interest, but is unlikely to be of practical importance, since the concentration at which their growth begins to be depressed is about ten times normal plasma concentration

SUMMARY

The influence of fluorine ion added in concentrations of 1, 2, 4, 8, and 16 ppm to the culture medium on the growth *in vitro* of human amnion cells, T (kidney) cells and HeLa cells, was studied DNA content of the

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NETHERLANDS SOCIETY FOR PHYSIOLOGY AND PHARMACOLOGY

ABSTRACTS OF PAPERS READ UNDER THE AUSPICES OF THE SOCIETY AT THE EIGHTH FEDERATION MEETING OF MEDICAL-BIOLOGICAL SOCIETIES, GRONINGEN, APRIL 6–7, 1967

E M Aarts, *Stimulation of the glucuronic acid pathway by drugs*
Department of Pharmacology, University of Nijmegen

A variety of drugs have been reported to stimulate the L-ascorbic acid synthesis in rat liver Evidence that this phenomenon concerns stimulation in the glucuronic acid pathway, arises from the finding that the administration of barbital, chloretone or 3 methylcholanthrene to rats stimulates the conversion of D hexose C^{14} to labeled D glucuronic acid, L-gulonic acid and L-ascorbic acid It appears that the said drugs stimulate the glucuronic acid pathway at some step prior to the conversion of D glucuronic acid D glucaric acid is a metabolic product of D glucuronic acid In view of these findings an enhanced excretion of D glucaric acid would be expected to occur during stimulation Barbital enhances the excretion of D glucuronic acid L-ascorbic acid and D glucaric acid in rats, chloretone enhances L-ascorbic acid and D-glucaric acid excretion, but 3 methylcholanthrene is found to enhance the L-ascorbic acid excretion only (BURNS and CONNEY, 1966)

In our experiments a single dose of thiopentone, DDT, amidopyrine, phenylbutazone or nikethamide, administered to rats, has no influence on the excretion of D glucaric acid, while at the same time the excretion of L-ascorbic acid is enhanced A single dose of DDT or nikethamide does not enhance the excretion of D glucuronic acid

During treatment of rats with cumulative doses of D glucurono lactone the increase of D glucaric acid production displays a pattern different from the increase of L-ascorbic acid production At low doses there is a considerable increase of D glucaric acid production, but not of L-ascorbic acid production Furthermore the stimulators barbital, nikethamide, phenylbutazone and amidopyrine enhance the excretion of L-ascorbic acid but not of D glucaric acid in rats loaded with D glucuronolactone (AARTS, 1967)

The results may indicate that the L ascorbic acid pathway and the D glucaric acid pathway have their own D glucuronic acid pool It is supposed that the L-ascorbic acid pathway together with the D glucuronic acid generating system form one physiological sequence of enzymes

Since the physiological significance of the D-glucaric acid pathway is not established (SADAHIRO *et al*, 1966), the possibility

could still be recognized as basophylic segments rich in nuclei

In the diaphragm the necrosis was much more abundant in the vicinity of the end plates than in the peripheral parts of the fibres Moreover, in the diaphragm the muscle fibres were more affected than in the other muscles Rats which had been given an LD_{10} of soman, which has no effect on the end plate function, had no muscle necrosis No necrosis was observed in the other organs examined

An oxime was injected 1, 4 or 8 h after DFP to investigate whether the development of necrosis could be prevented by reactivation of cholinesterase Each rat received 150 mg/kg P_2S i p In those rats which received the oxime one hour after DFP less necrosis was found the next day

All evidence so far points to the hypothesis that a relation exists between inhibition of the cholinesterase at the end plate and the muscle fibre necrosis

C M Ballintijn, *The influence of proprioception upon respiratory neurons in the medulla oblongata of fishes*

Zoological Laboratory of the University of Groningen, Haren (Gr)

In the past, it has been shown that several brain stem respiratory neurons are able to generate rhythmic activity, also when rhythmic proprioceptive stimuli are absent VON BAUMGARTEN (1956), VON BAUMGARTEN and KANZOW (1958) and COHEN and WANG (1959) used succinylcholine, which transitorily lames mammalian muscles, to exclude rhythmic proprioception Their main point is, that the respiratory neurons under observation then continue to fire rhythmically

Only scant information is given on the influence exerted by proprioceptive stimuli VON BAUMGARTEN and KANZOW (1958) recorded a change in the activity rhythm of cells during paralysis COHEN and WANG (1959) observed rhythmic inspiratory discharges in the region of the mesencephalic root of the trigeminus nerve, which became continuous during succinyl paralysis They interpreted these potentials as proprioceptive impulses from the jaw, which are known to be carried in the mesencephalic trigeminus

Only VON BAUMGARTEN and SALMOIRAGHI (1962) worked with

that the responsible enzyme in this pathway may have a function not related with physiological D glucuronic acid metabolism cannot be excluded The following mechanism for the different effect of stimulators is proposed stimulators initiate an enhanced D-glucuronic acid formation with consequences for the L ascorbic acid pathway and the D glucuronic acid L xylulose cycle If a markedly enhanced D glucuronic acid formation is initiated, an outflow of D glucuronic acid to the D glucaric acid pathway (barbital, chloretone) and to the extra cellular space (barbital) also occurs

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A Th Ariens and R M J van Benthem, *Necrosis of striated muscle fibres in the rat after injection of cholinesterase inhibitors*

Medical Biological Laboratory of the National Defence Research Organization TNO, Rijswijk Z H

Albino rats of 200 g were intravenously injected with the LD_{50} of DFP, tabun, S_{27}, S_{36} or S_{40} The S compounds are systox derivatives The diaphragm and the intercostal gastrocnemius and psoas muscles were studied at two hour intervals up to 12 h and subsequently each day, during 10 days after the injection The material was fixed in 10 % formalin solution Sections, 7 μ thick, were coloured with haematoxylin eosin or with the PAS technique Other organs examined were lungs, brains, kidneys, heart and spleen This work was done in conjunction with the investigations by Wolthuis and Meeter described in their abstract

The microscopic examination of the muscles revealed a process of partial necrosis of the fibres followed by complete regeneration The first abnormalities appeared after 2 h and consisted in an uneven swelling of the sarcoplasm with eosinophylia After 4–6 h the first leucocytes were seen and 10–12 h after the injection the partial fibre necrosis was fully developed After 3–4 days the first new striated fibrils were visible After 10 days the necrotic parts

could still be recognized as basophylic segments rich in nuclei.

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excitation threshold, but when a small continuous proprioceptive signal is added to it the threshold will be reached periodically and the neurons fire in rhythmic bursts With a large proprioceptive input the excitation is always above threshold and the cell is active without interruption

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A J J Beerens, *Discharges from primary afferents in the utricular nerve in the cat*

Dept of Neurophysiology and Ear, Nose and Throat Clinic of the Wilhelmina Gasthuis, University of Amsterdam

The purpose of the experiment is the investigation of the response-pattern of the utricular macula in mammals, in comparison with the findings in lower vertebrates (LOWENSTEIN and ROBERTS, 1950, VON HOLST, 1950, ROSS, 1936, SCHOEN, 1957)

After performing a partial labyrinthectomy (section of the ampullary nerves to the horizontal and anterior vertical semi-circular canals) on the left side, a microelectrode is introduced into the internal acoustic meatus on that side

While recording from the superior vestibular nerve solely active fibres from the utricular nerve are encountered

In the normal (horizontal) position a rather constant resting-discharge at a rate of some 20 to 40 impulses/sec (50 to 200 micro V) is recorded

Occasionally the resting discharge consists of more or less irregular bursts When altering the position of the cat (slow tilting towards the left) the discharge frequency is found to increase sharply to a level between 80 and 120 impulses/sec As long as the animal is kept in this tilted position, the discharge frequency remains constant at this higher level

When the animal is brought in the normal position again, the discharge immediately returns to its original pattern

Change of position towards the right (tilting) provokes a decrease

succinylcholine in fish They came to the conclusion that, contrary to its effect in mammals, the paralysis is irreversible

The respiratory neurons described by these authors all continue their rhythmic activity after succinyl injection until the end of the experiments

Artificial abduction or adduction of the opercula both in normal and in paralyzed animals did not influence central respiratory activity, except in a few cases where the imposed movement was abnormally great A sudden injection of 10 cc of water into the mouth sometimes resulted in changes in the firing pattern of the respiratory neurons under observation

The present report deals with the choice of a suitable relaxant and with the influence of proprioceptive input upon the respiratory centre

As the firing pattern of the neuron after recovery from paralysis serves as a control and in my experiments the irreversible action of succinylcholine was clear the properties of other relaxants were investigated Although the dose is rather critical, flaxedyl (gallamine) appeared to produce a paralysis that lasts for about half an hour, which the animal survives if the gills are irrigated artificially

The experiments with flaxedyl lead to the conclusion that, depending on their reaction to paralysis, the neurons in the respiratory centre can be divided into three categories One group of cells continue firing rhythmically during the period of complete immobility Evidently these cells are part of the structure which generates the respiratory rhythm or primarily depend upon it for the generation of their own activity The second group stops firing to resume its activity only when respiratory movements start again This group of cells consequently must be a part of, or mainly dependent upon the proprioceptive system Finally the third type of neuron is silent during paralysis Proprioceptive input as artificial abduction of the lower jaw, however causes them to resume their activity, which is continuous if the abduction is great but consists of rhythmic bursts if the mouth is slightly opened This remarkable behaviour can be explained as follows the cells are connected to the structure generating the respiratory rhythm and also to the proprioceptive system, either directly or via the neurons of group two The rhythmic input alone is, however, below their

stimulus, applied exactly at the same moment as before, caused a less deep inspiration, followed directly by a second one This difference appears to depend on the activity of the expiratory centres influencing the frequency of breathing Evidence for this hypothesis is the fact that this doubling of the induced inspirations always disappeared after bilateral vagotomy.

In some dogs the induced inspirations were preceded by some expiratory activity, the effect being the stronger the earlier in the expiratory phase the stimulus was given

These effects probably depend on the excitatory condition of the respiratory centres We hope this method will offer a new approach to the problem of rhythmicity of breathing

R A Binkhorst and J A Vos, *Short term exercise and the effect of warming up*

Department of Physiology, University of Nymegen

In continuation of previous experiments (BINKHORST and VOS, 1966) one subject (aerobic capacity of 4 8 l per minute) performed exercise on a bicycle ergometer during one minute, at loads varying from 100–650 Watt (612–3965 kg/min), with 70 rpm In one series the work was preceded by a warming up period of 10 min bicycling at 0 Watt, the exercise of the second series was preceded by a warming up period of 10 min bicycling at 155 Watt Oxygen consumption (Douglas bag, Haldane), ventilatory data, heart rate, pH and lactic acid were measured

The results of each experiment were expressed in cumulative curves in order to determine the total amount of transported oxygen ventilated volume and sum of heartbeats in the first minute of exercise, after the warming up period

RESULTS

1 All the parameters ultimately reached values which were 70–90 % of those reached at maximal oxygen consumption tests

2 When the total amount of oxygen transported and the total amount of heartbeats were plotted against the respective workloads, a distinct tendency for a levelling off of the parameters, with an increasing load, was found

of the resting frequency In any given position during lateral tilt the discharge frequency is indicative of that position

Recording from a primary afferent utricular nerve fibre shows a distinct frequency modulation of the discharge during tilt in the transversal plane

These findings in the cat are in good accordance with those in lower vertebrates and give support to BREUER s hypothesis that stimulation of the otolith organ is effected by the shearing force of the otolith *i e* in the transversal plane by outward displacement

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J A Bernards and J F Sistermans *Transient changes in lung ventilation by brief stimulation of the carotid bodies in the dog*

Department of Physiology University of Nijmegen

Spontaneous ventilation of dogs anaesthetized with chloralose urethane was recorded by means of a pneumotachograph The arterial chemoreceptors of the carotid bodies of both sides were stimulated simultaneously by injecting 1 ml of blood previously equilibrated with a gas mixture containing 7 % O and 10 % CO in N_2 near the arterial bifurcation within one half to one second

Injection in the expiratory phase caused an immediate deep inspiration after a latent period of about one second the volume of this inspiration being larger as the injection was given later Late injections caused little or no change in frequency early injections however produced an increase in frequency

Stimulation during inspiration increased the volume of this inspiration to some extent followed by an immediate second breath of smaller volume in most cases

In some experiments the induced inspirations were not very deep but they were always followed directly by a second inspiration In one experiment a stimulus during expiration was followed by one deep inspiration without change in frequency Half an hour later when the spontaneous frequency of the dog was higher a

specially shaped resistance body with holes straightens the relation between flow and output

The air flows from the flowmeter through a hosepipe and an inlet valve to the mask The expired air flows from the mask through an exhaust valve and a second hosepipe to the mixing drum

The mixing drum has a content of about 5 dm^3 The inlet is tangential, this keeps the air in a circular movement and gives an excellent mixing action The outlet is central and provided with an exhaust valve

The expired air sample in the mixing drum diffuses to the oxygen analyser

The oxygen analyser is of the polarographic type and has two cells for measuring the difference in oxygen concentration between inspired and expired air Both cells are at the same temperature The cell in the expired air measures the unknown concentration and is shunted by a resistor The other, the reference cell, is shunted by a servo controlled resistor in such a way that the voltage on both cells is equal The current ratio and also the resistor ratio is linearly related to % O_2 The accuracy is better than 0 1 % O_2

Two potentiometers are coupled to the servosystem One gives a signal proportional to ΔO_2 The other is fed with the signal from the flowmeter and gives a signal proportional to $\Delta O_2 \times$ flow After integration this gives the oxygen consumption

A C Bobbert, *Neuro-muscular transmission in the intact frog*

Department of Physiology, University of Leiden

In frogs w

not every im

and contract

the neuro muscular transmission ratio is physiologically less than one

This transmission ratio decreases when the firing rate of the motoneurones increases and further depends on their firing pattern

These observations strongly suggest that in the living frog depletion and mobilization of acetylcholine play an important part in neuromuscular transmission

3 However, the total ventilated volume increased more than linearly when plotted against the respective workloads
4 Taking the warming up into account it was shown that there was only a small difference in the determined values at the high loads For the oxygen transported, it could be calculated that at about 680 Watt it would have been the same, independent of the difference in warming up This suggests that a warming up at 0 Watt would have been sufficient for the work at high loads However, personally the subject preferred a warming up at 155 Watt Furthermore, he could not fulfil the work at 540, 600 and 650 Watt when preceded by a warming up at 0 Watt, it was impossible to have the muscles go on working probably because of a low *p*H in the muscles
5 The results presented in 1, 2 and 4 suggest that the limiting factor for the levelling off is probably the amount of stimuli to the heart at the onset of the exercise after the warming up Clearly ventilation (3) does not follow this closely, it might be that here other stimuli also play an important part in increasing ventilation at the beginning of exercise
6 It can also be concluded that it is not the ventilation as a mechanism but the transporting and delivering system that sets the limit for a larger oxygen uptake (1)

REFERENCE

BINKHORST R A and J A Vos Metabolism and respiration during severe muscular exercise (to be published in Acta Physiol Pharmacol Neerl)

J Bleeker and M Hoogendoorn, *A portable apparatus for continuous measurement of oxygen consumption during work*

Netherlands Institute for Preventive Medicine T N O, Leiden

This apparatus mainly consists of a flowmeter mask with valves mixing drum and oxygen analyser

A simple mechanical type of flowmeter has been designed for measuring the respiration volume The electrical output is directly proportional to the flow and is not influenced by movements of the person examined

The flowmeter has a rectangular cavity in which a valve is hinged This valve is balanced A spring delivers the counter couple A

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F I M Bonke L N Bouman and H E van Rijn,

The length of the post extrasystolic interval after atrial premature beats

Department of Physiology University of Amsterdam

In the isolated spontaneously beating right atrium of the rabbit, premature beats were elicited by electrical stimulation The intervals between the extracellularly derived atrial complexes were measured continuously with two electronic interval timers The frequency of the spontaneously beating atrium was found to vary only slightly A maximal standard deviation of 0 5 % was found from 60 samples of 10 succeeding normal intervals out of 20 experiments (confidence level 95 %)

After an atrial premature beat the interval (post-extrasystolic pause) is in most instances longer than the normal interval Two hypotheses have been put forward to explain the length of this post extra systolic pause ENGELMANN (1897) followed by WENCKEBACH (1903) assumed a decrease in the conduction velocity after the stimulus ECCLES and HOFF (1934) however assumed that the discharge in the S A node after the premature beat is delayed as they found a relation between the length of the curtailed cycle and the duration of the post extra-systolic pause

In the isolated rabbit heart we observed a similar relationship Furthermore we obtained experimental evidence that several factors determine the actual length of the post-extrasystolic pause

1 a decrease in rate of diastolic depolarization of the pacemaker fibres (BIERSTEKER *et al* 1966)
2 a shift of the pacemaker in the S A node area
3 changes in the conduction velocity of the atrium and S A node

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F H Bonjer, *Application of a portable apparatus for continuous registration of oxygen intake*

Netherlands Institute for Preventive Medicine TNO, Leiden

As early as 1911 DOUGLAS determined the oxygen intake in different forms and intensities of work His method consisted of collecting the expired air in large bags, after which the volume and the composition of the expired gas were determined This method is still frequently used Newer methods, as described by KOFRANYI and MICHAELIS (1941) and by WOLFF (1958) are based upon direct measurement of the volume of expired air by means of a portable dry gasometer, or a pneumatachograph, respectively, in both methods part of the expired air is sampled for gas analysis

The methods mentioned above share the following drawbacks

1 Between measuring periods large or small bags must be changed, valves or stopcocks must be manipulated, and often readings must be done,
2 the duration of the measuring period is limited especially if Douglas bags are used,
3 variations in oxygen intake during one and the same measuring-period are levelled out

The apparatus, as described by BLEEKER and HOOGENDOORN, has been developed with the aim to meet these drawbacks The volume of the inspired air as well as the oxygen percentage of the expired air are measured directly The results are transduced to an electrical signal and transmitted by cable or by means of a telemetering device In this way continuous registration of the oxygen uptake is obtained, which may last as long as the mouth-piece and the noseclip can be worn without too much discomfort A nose mask prolongs this period considerably

The method is applicable as well, if it is impossible to get near the subject It is not necessary to split complex tasks into components if the oxygen intake for the different parts of the task must be known

To callibrate the apparatus a Douglas bag is attached to the "exhaust" Tests during rest and during various occupations show the accuracy to be equal to that of existing methods

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G A Charbon and R S Reneman, *Parathormon a selective vasodilator*

Department of Pharmacology and Department of cardiovascular surgery, University of Utrecht

In a previous paper (CHARBON, 1966) data were presented on a diuretic and hypotensive action of parathyroid extract (PTE) Both effects occurred shortly after injection, i e within half an hour and within 5 min respectively

The cause of the hypotension induced by PTE was further investigated The blood flow to various regions was measured in anaesthetized dogs with a square wave electromagnetic flow meter PTE appeared to enhance selectively the arterial flow through the liver and through the renal arteries within 1 min The effect was log dose dependent Maximal increase of the flow was obtained with 3 2 μg of parathormon i v per kg At this dose level no change was observed in the minute volume of the heart No change either was seen in the flow through the common carotid arteries or the superior mesenteric artery, nor in the blood supply to spleen, pancreas and stomach The flows through the femoral, the inferior mesenteric and the vertebral arteries were decreased by PTE Arterial and venous pressure as well as the heart rate remained unaltered Oxygen saturation, carbon dioxide content, base excess, pH nor standard bicarbonate of the arterial blood were influenced by PTE

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CHARBON G A, A diuretic and hypotensive action of a parathyroid extract Acta Physiol Pharmacol Neerl 14, 52 (1966)

L N Bouman and F I M Bonke, *Impulse conduction through the sino auricular node*

Department of Physiology, University of Amsterdam

The conduction velocity of impulses was measured around and in the S-A node of the isolated right atrium of rabbit hearts The auricle was electrically paced by shocks with a duration of 0 5 m/sec, a voltage of 1 V above threshold and a frequency of 10 % above the spontaneous heart frequency Numerous micro-electrode im palements were made Between two impalements the micro electrode was moved 0 5 mm perpendicular to the terminal sulcus or 1 mm parallel to it The intervals between the pacing stimulus and the fibre discharge were measured with an electronic interval timer, which was started by the stimulus and arrested by the fast rise of the differentiated action potential From these intervals the conduction velocity was calculated

The most important conclusions from our experiments are

1 In the atrium the conduction velocity is highly variable, the average velocity was about 1 m/sec
2 In the crista terminalis the transverse conduction velocity is only about 40 cm/sec
3 In the S A node, away from the crista, the conduction velocity falls across a distance of 3 mm to only 1 cm/sec or less
4 Further away from the crista the fibres are activated earlier, apparently the activation travels faster above and below the node than through it (PAES DE CARVALHO *et al*, 1959, SANO and YAMAGISHI, 1959)
5 The conduction velocity inside the S A node is highly dependent upon the frequency of the atrial stimulation Intracellular records from a fibre in the centre of the node showed that when the frequency is stepwise increased from 2 8 c/sec to 4 c/sec, the conduction time initially increases slowly When, however, the frequency exceeds 3 8 c/sec the conduction time will quite suddenly increase more than fourfold
6 This change in conduction time following the frequency in crease is partly due to a concomitant decrease in the rate of rise of the nodal action potential

of aliesterase towards tributyrine which in rat plasma is 30 times lower than in guinea pig plasma

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V Claassen and J E Davies, *Potentiation by tricyclic antidepressants of the weight loss caused by amphetamine in rats*

Pharmacological Laboratories, N V Philips Duphar, Weesp

A number of effects of amphetamine have been found to be potentiated by tricyclic antidepressants, *e g* increase of locomotor activity stereotypic behaviour, hyperthermia etc It was investigated whether the same potentiation occurred with regard to the anorexogenic properties of amphetamine

Male albino rats individually housed were pre trained during three weeks to eat food during a 6 hours' period during the day For the test the drug (3 dosages i p) and dl amphetamine sulphate (2 4 and 8 mg/kg s c) were injected 45 min before the normal time of giving food The food consumption, water consumption and body weights were then measured every hour during 6 h The mean increase in body weight of 6 rats per dose level as obtained with desmethylimipramine is shown in Fig 1 As can be seen from these graphs a clear potentiation of the effect of amphetamine on body weight occurs at the dosages of 0 3 1 0 and 3 0 mg/kg desmethylimipramine The extent of potentiation is dependent on the dose of amphetamine used and is most pronounced at a dose of 4 mg/kg amphetamine sulphate

In all experiments the effects on food and water consumption showed a very comparable path during the feeding period

Nortriptyline also gave a strong potentiation of the amphetamine effect but was found to be about 25 times weaker than desmethyl imipramine when a comparison of dose response curves was made

A number of non antidepressant psychotropic drugs was also investigated in this test Haloperidol (0 3 and 1 mg/kg i p) gave itself an antagonism of weight gain, but in combination with

P. J. Christen and E. M. Cohen, *Binding of ^{32}P sarin to esterases and other proteins in plasma from rat, man and guinea pig*

Medical Biological Laboratory of the National Defence Research Organization TNO, Rijswijk Z.H.

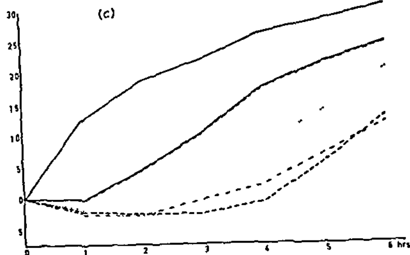
In blood plasma the potent acetylcholinesterase inhibitor sarin reacts with various esterases and with an enzyme which catalyses its hydrolysis (CHRISTEN and COHEN, 1963; CHRISTEN and VAN DEN MUYSENBERG, 1965). In experiments using Sephadex gel filtration it was found that during incubation of rat plasma with 0.01 mM ^{32}P sarin 35 % of the added ^{32}P was bound to protein. In human plasma this figure was 7 % and in guinea pig plasma 11 %. The esterase activities were inhibited after this treatment.

Using a microgel electrophoresis technique in combination with autoradiography the nature of the plasma proteins to which ^{32}P had become attached was studied. It was found that in human plasma all detectable radioactivity coincided with the albumin fraction, while in rat plasma the ^{32}P was found at the same spot as the aliesterase. In guinea pig plasma the radioactivity was nearly equally distributed over the albumin and the aliesterase. The amounts of ^{32}P bound to cholinesterase were too small to be detectable under our experimental conditions.

When after the incubation of rat plasma with 0.01 mM ^{32}P sarin the pH of the incubation mixture was lowered to 5.5, only 5 % of the added ^{32}P remained attached to the proteins. After this treatment no detectable radioactivity coincided with the aliesterase activity. It was found that the inhibited aliesterase was reactivated. The binding of ^{32}P to human plasma proteins is not influenced by a decrease of the pH of the plasma-^{32}P sarin incubation mixture to 5.5. Human plasma contains no aliesterase.

Our results indicate that plasma aliesterase inhibited by reaction with sarin is readily reactivated by lowering the pH to 5.5. From the amount of ^{32}P released from the enzyme at pH 5.5 a concentration of catalytic centres in rat plasma of 3 μM was calculated.

Data derived from experiments published by MYERS (1952) also suggest the presence of high concentrations of aliesterase in rat plasma. When tributyrine was used as a substrate for aliesterase a much higher activity was found in guinea pig plasma than in rat plasma. Our experiments demonstrate a catalytic centre activity



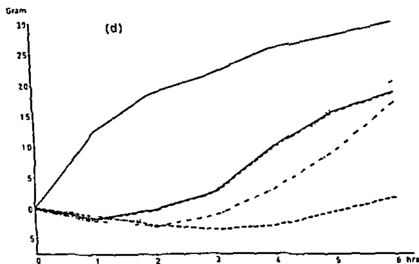
c) —— Blank

Control amphetamine 4 mg/kg

+ 0 3 mg/kg desmethylimipramine

+ 1 0 mg/kg "

+ 3 0 mg/kg "



d) —— Blank

Control amphetamine 8 mg/kg

+ 0 3 mg/kg desmethylimipramine

+ 1 0 mg/kg ,

+ 3 0 mg/kg ,

Fig 1

Increase in body weight during feeding period interaction between amphetamine and desmethylimipramine

a) —— Blank
▬▬ 0 3 mg/kg desmethylimipramine
1 0 mg/kg ,,
- - 3 0 mg/kg ,,

b) —— Blank
▬▬ Control amphetamine 2 mg/kg
+ 0 3 mg/kg desmethylimipramine
- - + 1 0 mg/kg ,,
+ 3 0 mg/kg ,,

mouse The hypothermia which was seen in the rat and the mouse after high doses could be a pre shock effect

CHANGES IN WHITE BLOOD CELL COUNT

In ruminants, horse, dog and probably in cat and swine leukopenia reached a maximum after 1 to 2 h The degree of leukopenia was positively related to the dose of pyrogen For the species studied, the following list of diminishing sensitivity can be made rabbit, horse dog, ruminants, cat and swine This result shows a rather good correlation with the fever effect of the pyrogen used, which makes it probable that these effects are related in some way

Although the gastro intestinal effects in different animals were not the same, some differences in sensitivity between the species were seen (hypomotility of the rumen, vomiting, diarrhoea)

It is concluded from the results that the different species appear to have a different sensitivity to pyrogens and that the following list of diminishing sensitivity can be given rabbit, horse, goat, dog cattle, sheep, cat, swine, chicken, mouse, rat, and guinea-pig

D O E Gebhardt, *The influence of 5 fluorouracil and of aminopterin on limb regeneration of Ambystoma mexicanum A comparative study*

Rijks Instituut voor de Volksgezondheid, Utrecht

Both aminopterin and 5 fluorouracil inhibit the *de novo* synthesis of thymidine Previous work by GEBHARDT and FABER (1966) had shown that

1) Oral administration of 100 mg/kg aminopterin on one of the first fourteen days after amputation through the humerus could retard the process of regeneration three weeks
2) Polydactylism occurred in 30 % of the regenerates if a single dose of 100 mg/kg aminopterin was given on one of the first eight days of regeneration
3) Administration of aminopterin on the fourteenth day after amputation yielded oligodactylism in 100 % of the regenerates

It has now been established that

1) By increasing the dose of aminopterin from 100 mg/kg to

amphetamine it inhibited the amphetamine weight loss Meprobamate (200 mg/kg p o) and *librium* (10 mg/kg i p) *did not* influence the amphetamine effect Cocaine (3 mg/kg i p) gave an indication of weak amphetamine potentiation

From the results obtained it is apparent that the anorexogenic effect of amphetamine can be potentiated by antidepressants As this interaction can come about in various ways – some of which are not strictly related to the central effects of the antidepressants, *e g* a decrease in rate of metabolism of amphetamine – this potentiating effect has to be interpreted carefully

J Frens and A S J P A M van Miert, *Effects of bacterial pyrogens in different animal species*

Institute of Veterinary Pharmacology and Toxicology University of Utrecht

In the present report the results are presented of a study dealing with the actions of a single pyrogen preparation in 12 different animal species A single batch of E coli 0111B4 lipopolysaccharide (Difco Laboratories) was used

BODY TEMPERATURE

In most species the temperature rose after a certain latency time Both latency and temperature rise were related to the dose of lipopolysaccharide Small doses gave monophasic temperature curves In some animals biphasic temperature curves were observed after moderate or high doses of pyrogen The temperature effect was calculated in two ways

1 The average of the individual temperature maxima in an experimental group
2 By means of a "fever index ", this being the area in cm² under the fever curve during 6 h of measurement

The dose level giving an average temperature reaction of +0 6 °C (12 cm² *respectively*) *was calculated The dose range for this* reaction was for the rabbit and the horse between 0 0001 μg and 0 001 μg/kg body weight intravenously For the goat and the cow it was between 0 001 μg and 0 01 μg and for the dog, sheep cat and swine between 0 01 μg and 0 1 μg/kg body weight intravenously There was no fever reaction in the rat, guinea pig and

Subjects were 10 Wistar rats, 6 months of age, conditioned daily after 16 h of water-deprivation to press a button and to be rewarded with a drop of water

PROCEDURE

PERIOD I

On 5 successive days the learned responses were recorded at 10 a m, spontaneous activity at 2 p m during 5 min each No significant correlations were found between the parameters of free and learned responses

PERIOD II

On 6 successive days, two groups of 5 rats were administered alternately either a dose of the pharmacon or a placebo in an emulsion by a stomach tube

1 h, 2½ and 6 h after drug administration a check was made of learned, free and free behaviour respectively

PERIOD III

In period III, however, the sequence of behaviour-checks was "free", "learned", "free"

RESULTS

2-ethylcrotonylurea causes

1 decrease of the frequency of (R) in non water-deprived rats (2½ and 6 h),
2 enhancement of the frequency of (R) in water-deprived rats (1 h)
3 shortening of the latency times of the first button pressing (1 and 2½ h),
4 no effect whatsoever on the frequency of learned responses

It is concluded that water-deprivation plus 2-ethylcrotonylurea significantly enhance the C N S activity

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FERGUSON, J T and F V Z LINN, Antibiotic Medecine and clinical Therapy, vol. 3 (1956)

250 mg/kg on the seventh day after amputation, the percentage of polydactylous regenerates was greatly reduced and the percentage of oligodactylous regenerates increased

2) 100 mg/kg aminopterin increases, whereas 100 mg/kg 5 fluorouracil decreases the incorporation of thymidine methyl H³ in the DNA of the nuclei of blastema cells
3) Both aminopterin and 5 fluorouracil induce a synostosis of humerus with the radius and ulna in 100 % of the regenerates
4) Administration of 100 mg/kg 5 fluorouracil on the fourteenth day after amputation retards the regeneration process about eight weeks and yields oligodactylous limbs or limbs with the normal number of digits Polydactylous limbs were never observed after treatment with 5 fluorouracil
5) Both regeneration and growth are retarded more than twelve weeks if a combination of 100 mg/kg 5 fluorouracil and 300 mg/kg thymidine is administered on the fourteenth day after amputation On the other hand the combination of 100 mg/kg aminopterin and 300 mg/kg thymidine does not retard regeneration any more than aminopterin alone

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GEBHARDT, D O E and J FABER J Embryol exp Morphol 16 143 (1966)

H van der Helm Hylkema, P Visser and A W Szafran,
Effect of 2 ethylcrotonylurea on free and learned behaviour of the rat

Department of Physiology, Division of Psychophysiology University of Amsterdam

Rearing (R) is a well known parameter of "free" or 'spontaneous" behaviour of the rat Its frequency is considered by LÁT (1963) as a measure of the unspecific C N S activity, being positively correlated with the speed of learning

The present experiment had a twofold purpose 1) to determine relations between frequency of (R) and two parameters of a learned Skinner response (S) *i e* frequency of button pressing as well as latency time between start and first response, 2) to study the effects of 2 ethylcrotonylurea (FERGUSON and LINN, 1956) on (R) and (S)

Differentiation of various types of spasmogens on guinea pig ileum

	In the presence of lachesine		After hemicholinium incubation		After procaine treatment		In the presence of hexamethonium	
Furtrethonium	0	(6)	76	(9)	95	(9)	98	(6)
Acetyl carbocholine"	0	(8)	2	(8)	7	(7)	106	(15)
Acetyl silicocholine	1	(6)	1	(9)	3	(12)	73	(20)
Acetyl glycol	2	(12)	0	(8)	0	(12)	77	(12)
Nicotine	1	(7)	2	(9)	6	(6)	2	(19)
$BaCl_2$	128	(7)	68	(10)	116	(9)	101	(8)

The contractions of the various spasmogens are expressed as mean percentage of the contractions under control conditions
The number of experiments is given in brackets

The possibility that the positive charge (δ^+), still present on the central carbon and silicium atom of both isosteres, is essential for their spasmogenic action, could be excluded by testing the so called "acetyl glycol", an ester in which the onium group is substituted by an OH group bearing definitely no positive charge "Acetyl glycol", too acts as a spasmogen and behaves in the differentiating tests in the same way as acetyl"carbocholine" and acetyl"silicocholine'

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MACINTOSH F C R I BIRKS and P B SASTRY, Nature 178, 1181 (1956)

P Th Henderson and E J Ariens, *Acetyl "carbocholine" and acetyl "silicocholine", directly or indirectly acting cholinergics?*

Department of Pharmacology, University of Nijmegen

The nitrogen free isosteres of acetylcholine acetyl"carbocholine" (3,3 dimethylbutylacetate) and acetyl"silicocholine" (trimethylsilylethylacetate), show a spasmogenic action on the isolated gut of the rat and the guinea pig Since the onium group, which seems to be necessary for a cholinergic action (ARIENS, 1964, 1966) is absent in these compounds the question arises whether the "cholinergic action" of these isosteres has to be regarded – as suggested by BURGEN (1965) – as a direct cholinergic action based on the interaction between the drug involved and the cholinergic receptors on the effector tissue, i e the smooth muscle tissue in the organ or whether it has to be regarded as an indirect cholinergic action based, for instance, on the liberation of endogenous acetylcholine or a protection of the endogenous acetylcholine against the inactivating acetylcholine esterase

The spasmogenic action of acetyl"carbocholine" and acetyl-"silicocholine" could be identified by testing these compounds under different conditions

1 In combination with an anticholinergic lachesine
2 After blockade of the acetylcholine synthesis by hemicholinium (MACINTOSH *et al* 1956)
3 *After blockade of the acetylcholine release by procaine* (FELDBERG and LIN 1949)
4 After treatment with a ganglionic blocking agent hexamethonium

The experimental results obtained are summarized in the following table

From a comparison of the spasmogenic behaviour of the isosteres of acetylcholine under the specific differentiating conditions with the spasmogenic behaviour of the directly acting cholinergic furtrethonium the ganglion stimulating nicotine and the mainly non cholinergic acting Ba ions it can be concluded that acetyl-"carbocholine" and acetyl"silicocholine" are to be considered as indirectly acting cholinergic, non nicotine like compounds the action of which is possibly based on a stimulation of the liberation of acetylcholine at the presynaptic nerve terminals

Some striking similarities with the distribution pattern after glucose UL-^{14}C suggest that labelled products occurring in the Krebs' cycle are involved

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F C Jager, *Vitamin E and poly unsaturated fatty acids*

Unilever Research Laboratory, Vlaardingen

In recent years, there has been a growing interest in the use of dietary fats with a high poly unsaturated fat content In practice this implies the use of vegetable oils having a high linoleic acid content Although it was known that the vitamin E requirement increases with increasing consumption of linoleic acid, the quantitative relationship between these two factors was not clear

In cases of vitamin E deficiency, the erythrocytes of rats show a strong spontaneous hemolysis *in vitro* It has therefore been investigated to what extent this phenomenon may serve as test method for the indication of the vitamin E requirement

Within a specific dosing range, there appeared to exist a negative linear relationship between the log-dose vitamin E (mg/kg food) and the percentage hemolysis This hemolysis *in vitro* (in NaCl-phosphate buffer pH 7 4, at 38 °C) reached a practically constant level after 4 to 5 h This level was higher for a diet with 37 cal % maize oil (made tocopherol free) than for a corresponding lard diet, the dose of vitamin E added to the diet being the same To prevent hemolysis *in vitro* 11 8 mg D α tocopherol acetate per kg food was required in the lard diet whereas in the case of the maize oil diet, 19 3 mg was required

For the rat, the conclusion can be drawn that in case of an increased linoleic acid consumption the vitamin E requirement increases by 0 1 mg D α tocopherol acetate per g linoleic acid This implies that vegetable oils with a high linoleic acid content by nature contain sufficiently vitamin E to meet this extra requirement

W Hespe and H Prins, *Autoradiographic investigations into the distribution of exogenous GABA in the mouse*

Research Department of the N V Koninklijke Pharmaceutische Fabrieken v/h Brocades Stheeman and Pharmacia, Amsterdam

(in collaboration with Dr E Roberts, City of Hope Medical Centre Duarte, California)

At 1, 2, 4, 8 and 16 h after i p administration of a 0 15 mg dose (5 μc) of GABA-UL ^{14}C to female mice the distribution of radioactivity was studied with a macro autoradiographic technique (ULLBERG, 1954)

One hour before GABA administration, part of the animals re ceived an i v dose of 25 mg/kg aminooxyacetic acid (AOAA), known as a strong inhibitor of GABA-transaminase (WALLACH, 1961) The established strong influence of AOAA on the distribution of radioactivity suggests a nearly complete inhibition of the GABA breakdown by AOAA, the distribution pattern after AOAA-treatment, especially at the early time intervals, being representa tive for unchanged GABA Assuming this we may consider the distribution of exogenous GABA as characterized by affinity to the intestinal wall, liver, kidneys (urine), hypophysis and vertebral and costal cartilage leading to a highly increased concentration in these organs and tissues as compared to that of the blood Only slight levels of radioactivity not exceeding that of blood are found in the remaining part of the organism There is no pene tration of radioactivity into the CNS which contradicts a recent postulate that GABA enters the brain more readily after adminis-tration of AOAA (VAN GELDER 1965)

Without AOAA pretreatment the distribution pattern of radio activity is governed by labelled GABA metabolites already at the first time intervals studied The liver is now far less radioactive than at the corresponding time intervals after AOAA treatment which indicates that this organ is the main centre of exogenous GABA metabolism and AOAA influence Contrary to GABA itself the radioactive metabolites show a high affinity to Harder's gland the salivary glands, especially the sublingual gland and other mucous glands in tongue, palate and larynx, to a lesser extent there is also an obvious concentration of radioactivity in the CNS

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GUTTMANN, L and D WHITTERIDGE, Brain 70, 361 (1947).
JONGE, M C DE, J A KORNELIS en JW VAN DEN BERG, Acta neurol. Scandinav, Suppl 20, 42, 155 (1966)

A J M Kits van Heyningen, F Huying and R B. H Schutgens, *Some properties of 1,2 bisphosphoenolpyruvate*

Department of Physiology, Laboratory of Biochemistry, Department of Medical Enzymology, University of Amsterdam

1,2 bisphosphoenolpyruvate (bis-PEP), has recently been isolated and identified from incubation media of rat diaphragms (KITS VAN HEIJNINGEN, 1965) The structural formula has been confirmed by organic synthesis and comparison of the natural and synthetic products An enzymic method of determination was developed upon incubation of media with acid Phosphatase (E C 3 1 3 2) at pH 6 6 or with alkaline phosphatase at pH 8 (E C 3 1 3 1) all bis PEP will be transformed into pyruvate which can be determined subsequently with lactate dehydrogenase Acyl phosphatase (E C 3 6 1 7) did not hydrolyse bis-PEP Incubation media consisted of Krebs Henseleit phosphate buffer with 0 9 I U Insulin/ml and 50 μg Adrenalin/ml added, as this appeared to increase bis PEP secretion into the medium by more than 45 % (KITS VAN HEIJNINGEN, 1966)

In these media the following points have been investigated

1) The influence of iodoacetate (1 mM) and sodium fluoride (1 mM) on the production of bis-PEP Neither inhibited respiration of the diaphragms and both inhibited glucose metabolism as judged from the amount of glucose left in the media after incubation Sodium fluoride inhibited bis PEP production to an extent which precluded enzymic determination Iodoacetate increased bis PEP concentration in the media by 60 % as determined in 3 series of experiments on 10 hemidiaphragm pairs

2) The presence of oxygen did not prove to be essential to the formation of bis PEP In two experiments under N2 the ^{14}C-glucose was added to the Warburg vessels after 10 min incubation There was no difference between the amount of labelled

M C de Jonge, M A Crommelin, J Weits, J A Kornelis and J W van den Berg, *The effect of electrical stimulation of the urinary bladder on the circulation and respiration of spinal transected dogs*

Laboratory of Medical Physics, University of Groningen

Clinical observations of paraplegic patients pointed out that reflex activity of the neurogenic bladder could induce cardiovascular reactions The most important symptom was a large increase in arterial pressure observed in patients with a complete spinal lesion at or above the segment Th_5, caused by a vasoconstrictor reflex in arms and legs (GUTTMANN and WHITTERIDGE, 1947)

With regard to the development of an electrical stimulator for the treatment of the neurogenic bladder (DE JONGE *et al*, 1966) we studied cardiovascular and respiratory reactions in 8 dogs with a complete spinal transection at levels between C_7 and Th_{10} During electrically induced excitation of the urinary bladder we recorded the e c g, femoral arterial pressure and peripheral blood flow, by means of photoplethysmography and strain gauge plethysmography The effect on respiration was studied by simultaneous determinations of respiratory volume and esophageal pressure

Our results indicate a significant increase in systolic and diastolic pressure in dogs with a spinal transection at or above the segment Th_3 This hypertensive reaction reached a maximum of 200/150 mm Hg and was caused by a considerable increase in vascular resistance in both upper and lower extremities In the same dogs we observed sinu auricular arrhythmia during electrical stimulation of the bladder In dogs with spinal lesions below Th_3, bladder excitation evoked only very slight alterations in blood pressure, without any influence on heart rate

Bladder stimulation had no effect on the respiratory total volume or airway resistance The only reaction was an increased respiratory rate in one dog with a spinal transection at the level C_7

On the basis of these experimental results it is to be expected that electrical stimulation of the urinary bladder of paraplegic patients with high thoracic or cervical spinal lesions may induce hemodynamic changes similar – at least qualitatively – to the reactions as described during bladder reflex activity

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GUTTMANN L and D WHITTERIDGE Brain 70 361 (1947)
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A J M Kits van Heyningen F Huijing and R B H Schutgens *Some properties of 1 2 bisphosphoenolpyruvate*

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to cause PGE_1 like effects on ADP induced platelet aggregation In a roughly quantitative assay and expressed on a dose-level these substances showed 5 1 25 and 1 % PGE_1 activity respectively

As aggregation of blood platelets is thought to play an important part in thrombogenesis and maybe even in the genesis of athero sclerosis the finding that PGE_1 and PGE_2 influence platelet aggregation seems to be very important

H L Knook and P E Voorhoeve *Analysis of antidromically evoked responses in the olfactory bulb of the rabbit*

Department of Neurophysiology University of Amsterdam

Laminar field potentials in the olfactory bulb evoked by anti dromic stimulation of the lateral olfactory tract (LOT) have been described previously (OCHI 1963 PHILLIPS *et al* 1963) The *laminated structure of the bulb is determined by the layer of the* mitral cell bodies which send their axons into the LOT and a primary dendrite upward into the glomerular layer

In the core of the bulb the evoked potential consists of an initial negative wave N_1 of about 2 msec duration and a positive wave P lasting for some 15–20 msec Early in the P wave a small negative hump N_2 can be detected and in the trough of the P wave a fairly large negative wave N_3

On withdrawal of the micro electrode the configuration of these potentials changes From a depth of about 0 70 mm upwards corresponding to the level of the mitral cell bodies the N_1 wave is preceded by a sharp positive inflection and has the properties of a propagated spike potential along the *primary dendrites* At exactly the same depth where the N_1 wave becomes diphasic the P wave reverses to a slow negativity with the same time course It is as yet not clear whether this wave is due to *active* hyper polarization of the soma *i e* to inhibition or to a delayed re polarization of the electrotonically depolarized secondary dendrites in the external plexiform layer (ECCLES *et al* 1966)

The N_2-wave has its optimum just below the mitral cell layer *and* seems to be due to activation of internal granule cells by recurrent mitral axoncollaterals The N_3 wave does not reverse and has its maximum amplitude in the external plexiform layer It can *only* be evoked when the stimulating electrode is on the

bis-PEP formed aerobically or anaerobically In conclusion bis-PEP formation does not require the action of glyceraldehyde 3-phosphate dehydrogenase The possibility of its formation via the pentose-phosphate cycle is supported by the poor labelling obtained with [1-^{14}C]-glucose in the medium (KITS VAN HEIJNINGEN, 1965) The nature of the inhibition by fluoride is as yet unknown

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J KLOOZE, *Influence of prostaglandins on ADP-induced platelet aggregation*

Unilever Research Laboratory, Vlaardingen

Prostaglandins have been demonstrated to cause smooth muscle contraction It is also known that under certain conditions, blood platelets are able to contract, owing to a protein fraction which in many ways is analogous to muscular acto-myosin When a platelet contracts, intracellular fluid containing among others adenosine diphosphate (ADP) is released, resulting in aggregation of the blood platelets induced by the ADP released On account of these facts it has been examined whether prostaglandins are able to cause contraction of blood platelets and consequently platelet aggregation

None of the prostaglandins examined induced platelet aggregation in citrated platelet-rich plasma (cPRP), as was measured by a turbidometric technique However, it appeared that PGE_1 inhibited platelet aggregation induced by addition of ADP to cPRP of rats, pigs and men PGE_2 stimulated platelet aggregation induced by addition of ADP in cPRP of rats and pigs, but it was inactive in human cPRP Dose response curves showed that PGE_1 and PGE_2 were already active in doses as low as 10^{-3} to 10^{-2} μg/ml

The effect of PGE_1 and PGE_2 was found to be very specific as a great number of prostaglandins with very small differences in chemical structure appeared to be inactive Only three related substances, *viz* PGE_1-217, dinor-PGE_1 and an iso-PGE_1 were found

effect relation that phenobarbitone acts in more than one way on the metabolism of total brain RNA

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MCILWAIN, H M, R RODNIGHT, Practical Neurochemistry London, Churchill (1962) Chapter 6

R J M de Leeuw, Miss M L Hamelink, K A Foest and B F Visser, *Significance and measurement of erythrocyte-pH*

Lungfunction Laboratory, State University Hospital, Utrecht

The intracellular pH of red blood cells (pHi) is an important factor for the situation of the Hb HbO$_2$ equilibrium, and therefore for the oxygen transport function of the blood This function is of paramount importance, and is safeguarded against disturbance owing to coarse variations of the pH in three different ways by the high concentration of protein in the erythrocyte, by the Haldane Bohr effect, and also by the specific erythrocytary mechanism consisting of splitting off phosphate from diphosphoglycerate (and ATP[2]) in acidosis and combining phosphate with monophosphoglycerate (and ADP[2]) in alkalosis, the inorganic phosphate diffusing away to the plasma in exchange for bicarbonate (and chloride) in acidosis and vice versa in alkalosis

Planning an investigation of pHi in relation to the diphosphoglycerate mechanism in pathological acid base conditions, the first problem to be solved was working out a dependable method for determination of pHi

Direct measurement of pHi in cell hemolysate with the glass electrode + saturated calomel electrode is not only time consuming because of the preliminary manipulations (anaerobic and isothermal centrifuging anaerobic separating and freeze thawing), but also difficult on account of the high protein concentration at the hemolysate/saturated KCl boundary Nevertheless we came to reproducible pHi measurements, but, having the disposal of this direct method, we looked for a more simple, indirect procedure

pHi predicted from the blood-pH (pHe) appeared to have a

anterior olfactory nucleus and is presumably due to activation of a separate set of fibres originating in this nucleus and having synapses on the mitral cell secondary dendrites All these potentials can be explained by assuming a "closed field" in the core of the bulb and a laminated field above the mitral cell layer

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A van Langevelde and E L Noach, *Influence of phenobarbitone on RNA metabolism in rat brain slices*

Department of Pharmacology, University of Leiden

It is well known that the oxygen uptake of brain material, when measured *in vitro*, decreases under influence of phenobarbitone, while lactic acid production increases (BAIN, 1952) This has been attributed to inhibition of NAD(P)H oxidoreductase (GIUDITTA, 1963)

In our experiments rat brain cortex slices were incubated in Warburg vessels (McILWAIN, 1962) Inhibition of oxygen uptake is significant at phenobarbitone concentrations of 10^{-4} M and higher In the same experiments ^{14}C orotic acid was added to the slices in the vessels After incubation for 1 or 2 h total RNA in tissue and RNA radioactivity were determined (ADAMS, 1965) The uracil moiety in the RNA was found to be labelled and in all probability the cytosine moiety, too The specific activity (counts/min mg RNA) was about 1600 after 1 h, about 3700 after 2 h In the incubation solution free ^{14}C uracil could be detected along with ^{14}C orotic acid by thin layer chromatography Radioactivity and amount of uracil also increased with increasing time of incubation

Phenobarbitone was found to lower the specific activity of RNA in slices, incubated for 2 h, but total RNA content remained unchanged Phenobarbitone caused similar effects on free uracil in the incubation medium Unlike the decrease of oxygen uptake the decrease in specific activity is highly significant at 3×10^{-5} M phenobarbitone It becomes probable from the non linear log dose-

can be explained from the non linear stress-strain behaviour of the arterial wall material

We intend to study the influence of hydrostatic pressures on the propagation time with a larger number of subjects, to investigate whether this method is a useful tool to examine the stiffness of the large arteries in pathological cases

E Meeter and O L Wolthuis, *Anticholinesterase hypothermia in the rat and its use in the study of therapeutic agents*

Medical Biological Laboratory of the National Defence Research Organization TNO, Rijswijk Z H

Groups of albino rats of about 200 g were intravenously injected with various cholinesterase inhibitors It was found that DFP, soman, tabun, and a systox derivative with an uncharged molecule, S_{36} all produce a drop in rectal temperature of about 6° The lowest temperature is reached after 3 hours and recovery takes place in the next 25–35 h

During half hour periods rats were exposed to an environmental temperature 7° below the normal room temperature before, and at appropriate times after the injection of soman At the deepest point of the soman hypothermia the drop in body temperature resulting from the cold stress was 3 times as large as before the soman administration This demonstrates that the hypothermia is not a result of a shift in "set point" of the regulation mechanism, but is caused by a loss of regulating capacity

It was found that cholinesterase inhibitors with charged molecules the systox derivatives S_{27} and S_{30} do not produce hypothermia As LOMAX and JENDEN (1965) obtained hypothermia by intracerebral application of cholinomimetic drugs, the ineffectiveness of charged molecules can be explained by their inability to pass the blood brain barrier

Atropine sulphate intraperitoneally injected in doses ranging from 0 5 to 10 mg per animal a few minutes before the injection of the enzyme inhibitor, clearly counteracted the hypothermia, although even 10 mg did not completely prevent this phenomenon

The effects of various oximes, administered intraperitoneally one hour after the injection of the anticholinesterase, were studied P_2S (150 mg/kg) was found to have a slight anti hypothermic effect

standard error of the estimate (S E E) of 0 018 pH units [(n=243, pHe=7 406 (mean) ± 0 046 (S E), pHi=7 209 ± 0 032)]

Homolysed whole blood pH (pHh) would give a more reliable idea of pHi, because pHh largely depends upon pHi, erythrocyte contents having a much greater buffering capacity than plasma

The result of combined measurements of pHi, pHh and pHe was an acceptable S E E of pHi predicted from pHh, viz 0 009 pH units, prediction from pHh and pHe giving no further improvement (n=54, pHe=7 399 ± 0 043, pHh=7 259 ± 0 039, pHi=7 205 ± 0 033)

Th W van der Mark, L de Pater and Jw van den Berg, *Variation of the propagation time of the arterial pulse wave with hydrostatic pressure*

Laboratory of Medical Physics, University of Groningen

The propagation time of the arterial pulse wave is equated to the time between the R peak of the ECG and the beginning of the upstroke of the photoplethysmogram of the thumb The ECG and the photoplethysmogram are fed into an electronic pulse shaper The time between the output pulses of this shaper represents the propagation time These pulses trigger an electronic timer which is connected to a printer in order to record the propagation time in digital form

Since the pulse wave velocity depends among other things on the stress in the arterial wall the propagation time depends on the mean transmural pressure in the arteries This dependence can be investigated by measuring the propagation time at different positions of the arm varying the position of the hand from as far as possible below heart level to as far as possible above heart level

In our experiments we measured the propagation time of 11 healthy recumbent subjects with three different positions of the hand at heart level and in the extreme positions below and above heart level When we take the propagation time at the position of the hand at heart level as reference value, the propagation time in the high position is longer and in the low position shorter We found, converted to a level variation of 40 cm, in the high position a mean increase of 11 % (S D 2 %) and in the low position a mean decrease of 6 % (S D 1 %) The difference of these values

guinea pigs of the same breeding, one group serving as control (not subjected to exteroceptive stimulation) The test stimulus A concentration of DNCB just below the primary toxic/allergic threshold (which we previously established) The DNCB solved in acetone was applied to the skin of the animals on an exactly restricted surface of 2 cm² Exteroceptive stimulation a cylindrical cage with electrified grid (110 V, 1 mA)

Measure for skin reactions a Two intersubjective judgements b Photographic registration

RESULTS The Null hypothesis could be rejected in favour of the alternative hypotheses at a significance level of 0 01, on comparison of the intersubjective judgements Our photographs did not give enough possibility to differentiate between the groups

CONCLUSION In our experiment exteroceptive stimulation led to a lower threshold for a delayed type hypersensitivity as defined by us

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A S J P A M van Miert, *The effect of bacterial pyrogen and hyperthermia on rumen motility in the goat (preliminary communication)*

Institute of Veterinary Pharmacology and Toxicology, University of Utrecht

Ruminants suffering from infectious diseases often show a reduction in rumen motility, especially during the fever period This effect can also be observed after injection of infusion fluids, contaminated with pyrogens, or purified endotoxins from gram negative bacteria (VAN MIERT 1966)

In the present study 10 goats were given intravenous injections of different doses of a purified lipopolysaccharide (from E coli 0111B4 prepared by Difco) Special attention was given to body temperature, heart frequency and rumen motility (recordings were made using a system consisting of a water filled polyethylene tube with open end placed in the rumen combined with a straingauge

against DFP and soman As the soman-inhibited enzyme is almost completely "aged" after one hour, i e unable to reactivate, the P_2S effect on this hypothermia is not caused by reactivation of cholinesterase in the c n s MINA (150 mg/kg) is very effective *and rapid in action against DFP and clearly passes the blood-brain* barrier Toxogonin, given in a low dose (40 mg/kg) is strikingly effective against DFP but not against soman, which shows that it acts by enzyme reactivation and that it passes the blood-brain barrier In higher doses, up to 150 mg/kg, it has hypothermia aggravating side effects during the first two hours, before it demonstrates its beneficial action TMB_4 probably acts somewhat similarly

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LOMAX, P and D J JENDEN, Int J Neuropharmacol 5, 353 (1965)

P J G Mettrop and P Visser, *Exteroceptive stimulation as a factor in the induction and elicitation of delayed type hypersensitivity reactions to dinitrochlorbenzene (DNCB) in guinea pigs*

Division of Psychophysiology, Physiological Laboratory, University of Amsterdam

Recently the study of the direct influence of the nervous system on induction and elicitation of delayed-type hypersensitivity skin reactions has been denied a subject of discussion (A L DE WECK and J R FREY, 1966) In literature the proposition is often made that conflict-situations can potentiate allergic and sensitization reactions in man

In the present experiment we investigated the influence of exteroceptive stimulation on sensitization We expected that exteroceptive stimulation would lead to a lower threshold for a, delayed type hypersensitivity skin reaction to DNCB in guinea-pigs

NULL HYPOTHESIS $p = q$ Alternative hypothesis $p > q$, where p is the probability that an organism subjected to exteroceptive stimulation reacts to a test stimulus, and q is the probability that an organism not subjected to exteroceptive stimulation reacts to a test-stimulus

EXPERIMENTAL CONDITIONS *Organisms two groups of twenty*

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W F Muntjewerff, J A Kornelis and Jw van den Berg, *Acoustic properties of lungs and trachea*

Laboratory of Medical Physics, University of Groningen

This research was part of a series of experiments on the primary glottal sound The acoustic properties of the subglottal system strongly affect the vibratory pattern of the vocal folds

The experiments were performed on dogs of 15–20 kg body weight, after transection of the trachea anaesthetized by laughing-gas only If possible, respiratory actions were suppressed by hyperventilation, if not by succinylcholine administered with an infusion pump Before a measurement the respiration pump was disconnected from the trachea and the measuring apparatus was connected

This apparatus consisted of a vibrator and a microphone, both looking into a small space The system to be investigated, being connected to this small space, has an acoustic impedance dependent on the frequency The vibrator produces a gliding tone of constant volume velocity The pressure at the site of the microphone is therefore proportional to the impedance, for all frequencies

Calibration of the apparatus was performed by means of a stiff open tube length 30 cm The resonant frequencies and Q-values of this tube can be calculated The resonant frequencies of an open tube are related as 1 3 5

The subglottal system behaves like an open tube The resonances are slightly shifted, owing to the small radius of the tubes The wall properties also have some impact on the sound velocity and therefore on the resonant frequencies The Q values are also dependent on the radii of the pathways and on the wall properties

Some pharmaca were administered and the effects on frequencies

transducer and an electronic recorder) There was little or no reaction with 0 01 μg/kg body weight, with 0 025 μg and 0 1 μg, however, there was an average maximum temperature rise of 1 2 °C respectively 2 0 °C In the mean time the frequency and intensity of the rumen contractions were diminished by 44 and 56 % respectively 52 and 80 % The effect on body temperature and rumen motility reached its maximum 3 h after the injection During this period bradycardia was observed With 1 μg and 10 μg per kg body weight the temperature rise was 1 5 °C respectively 1 15 °C after ± 5 h The frequency and intensity of the rumen contractions diminished however within 60 min after injection, by 85 and 92 % respectively 87 and 96 % There was bradycardia during this period, thereafter tachycardia developed

The differences with high and low doses prompted us to make further studies

Hyperthermia also gives a reduction in rumen motility (intensity reduced by 84 to 93 %) On the other hand, the antipyretic novaminsulfonum (Novalgin[R] Hoechst) 50 mg/kg reduces the effect of 0 1 μg endotoxin on temperature and rumen motility

From the literature it is known that in the period of hyperthermia (ANDERSSON *et al*, 1963, ROBERTSHAW-WHITTOW, 1965), but also during endotoxin shock (HEIFFER *et al*, 1960, ROSENBERG *et al*, 1959), a marked release of catecholamines occurs There is hyperglycaemia during fever in ruminants (CAKALA, 1965, MULLENAX *et al*, 1966) In sheep, stimulation of the n splanchnicus gives a reduction in rumen motility (BRUNAND-NAVARRO, 1953)

In our experiments the intravenous infusion of epinephrine 1 μg/kg/min *gives a marked reduction in rumen motility* with bradycardia, often followed by tachycardia

It is therefore concluded that the reduction in rumen motility after an injection with endotoxin is possibly directly caused by a sympathetic stimulation and epinephrine release and indirectly by *the temperature rise elicited by this pyrogen*

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J F W Nuboer *Photometry in a wild rabbit*

Laboratory of Comparative Physiology, University of Utrecht

In order to measure the sensitivity of the light sense for a number of wave lengths, a wild rabbit is trained to choose between two stimuli a reference light and a test light The stimuli are simultaneously presented in an automatically working two choice apparatus Each stimulus appears as many times to the left as to the right of the animal in an unlearnable sequence

The animal is trained to respond to the reference light, a response to this light is continuously reinforced (food reward) This choice will be most difficult when the stimulus intensities of both lights match The rate of difficulty can be measured, considering the proportion of choices of the reference light

For each test wave length two series of experiments are performed One series is started at low stimulus intensity of the test light The animal thus learns to respond to the brighter reference

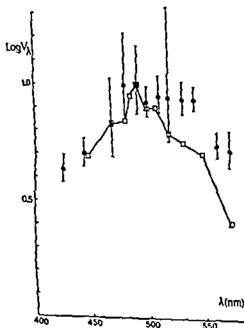


Fig 1

and Q-values correlated with the effects on the bronchial tree, found in literature

Changes of wall properties and tube radii were readily demonstrated with this method of investigation

E L Noach, E N Chin A Paw and P C M Goosen,
Quantitative aspects of false transmitter release by tyramine in reserpinized pithed rats after infusion of α-methyl noradrenaline

Department of Pharmacology, University of Leiden

In reserpinized animals, pressor effects of i v tyramine (Tyr) are normalized after an i v infusion of Noradrenaline (NA) (Burn & Rand) In previous work with pithed rats we demonstrated that rapid tyr tachyphylaxis is due to NA breakdown by mono-amino-oxydase After infusion of 25 μg α-methyl-noradrenaline (α-me-NA) which is not attached by MAO, tyr tachyphylaxis is absent The log dose-effect curve for pressor effects of Tyr (range 20–80 μg) after α-me-NA is exactly parallel to the curve of α-me-NA itself (range 0 125–1 000 μg) and significantly steeper than the curve of Tyr before the infusion Forty μg Tyr release the pressor equivalent of 0 150 μg α-me-NA Since the actual amount of α-me-NA released is obviously much smaller, the absence of tachyphylaxis for repeated doses of 40 μg tyr after infusion of 25 μg α-me NA is easily explained However, tyr tachyphylaxis after infusion of small amounts (1–2 5 μg) of α-me-NA is to be expected and did occur The possibility of receptor sensitization for tyr by α-me-NA could therefore be rejected

After desmethylimipramine (DMI) 3 mg/kg i v, the dose effect-curve of α-me-NA was shifted to the left, raising its pressor potency 4 5 times DMI after an α me-NA infusion reduced tyr pressor potency almost to zero Therefore, DMI does not sensitize adrenergic receptors for pressor amines Since it is known that DMI blocks uptake of catecholamines into tissue stores, the data presented indicate that about 80 % of a dose of α-me-NA is inactivated by tissue uptake before reaching adrenergic receptors Re uptake of α-me-NA, released by tyr, may contribute to the absence of tyr tachyphylaxis

time derivative of the left ventricular pressure It appeared that during the occlusion the ventricle contracts irregularly Owing to this irregularity no simple relation between the maximal ventricular pressure and the left atrial pressure could be observed In 13 dogs weighing from 15 5 to 32 5 kg the mean maximal pressure in the left ventricle was 355 mm Hg (S D 55) *i e* 280 % of the control value, and the corresponding maximal value of the derivative was 6100 mm Hg/s (S D 1620), *i e* 270 % of the control value

From these experiments the matching of the ventricle to the vascular system can be determined Then the ventricle is considered to be analogous to an electric generator with a constant voltage E and an internal resistance R_i, and when the input impedance of the vascular system is approximately represented by a resistance R_u the matching is given by the ratio R_i/R_u To get the correct pressure corresponding with E, the clamping of the aorta must occur exactly during diastole The ratio R_i/R_u can be calculated from the maximal pressure directly before and after the clamping For successful occlusions on 8 dogs R_i/R_u ranges from 0 28 to 0 69 Since coronary flow has been neglected the real values are still larger Hence we may conclude that the matching of the ventricle to the vascular system is quite good

R L Polak, *The influence of antimuscarinic drugs on synthesis and release of acetylcholine by the isolated cerebral cortex of the rat*

Medical Biological Laboratory of the National Defence Research Organization TNO, Rijswijk Z H

To be published in Journal of Physiology, Proc Physiol Soc , 17–18 March 1967

J Pool *Peripheral circulation and vasodilating drugs*

Department of Cardiology University Hospital, Leiden

In order to judge the effect of vasodilating drugs we made some measurements of the peripheral circulation, whereby we tried to distinguish between skin and muscle circulation

The methods we used were

light Next the radiance of the test light is made higher stepwise At each step the animal is permitted to choose 100 times The second series starts at relatively high stimulus intensity of the test light This time the animal thus learns to respond to the less bright reference light Next the radiance of the test light is lowered stepwise Meanwhile the radiance of the reference light (496 nm) is kept at $0\ 80 \times 10^{-2}$ $Wm^{-2}\ sr^{-1}$

Both series result in functions which describe the preference for the reference light in relation to the radiance of the test light This radiance corresponding to the intersection of both functions is supposed to be the stimulus intensity which equals that of the reference light The reciprocal of this value is used as a relative value of the spectral sensitivity

The results are represented as open squares (relative log scale) They are compared with results – filled circles – of another type of photometric measurement (optokinetic method in a tame rabbit, NUBOER, 1965) The maxima of both results – at 491 nm – are equalized

The greatest difference between the two results is found between the range of 500 nm and 570 nm It is suggested that this incompatibility might be a result of the stimulation in different parts of the visual field by each method, the two-choice method making use of the nasal parts of both visual fields, the optokinetic method making use of a more central part of one visual field

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L de Pater, J A Kornelis and Jw van den Berg,
Response of the left ventricle to a sudden occlusion of the aorta

Laboratory of Medical Physics, University of Groningen

In anaesthetized, open chest dogs the response of the left ventricle to a sudden, total occlusion of the aorta was determined The aorta was clamped during 5 to 10 sec by a forceps, so that the ventricle-contraction was virtually isovolumetric, since the output was reduced to coronary flow The following variables were continuously recorded the ECG, the pressure in the left ventricle, the left atrium and the aorta distal from the occlusion, and the

indication of the latter the renal excretion of phenol was used

The doses used were 10 and 20 mg of toluene per rat of about 250 g and 2 and 4 mg of benzene per rat These doses were calculated on the basis of the maximum allowable concentrations in industry

The dose of 20 mg of toluene diminished the amount of phenol excreted during the first eight hours after the administration of both doses of benzene The dose of 10 mg of toluene diminished the amount of phenol only after administration of the highest dose of benzene during the same period During later periods of collection of urine, no differences in phenol excretion were found among differently treated animals nor when these animals were compared with untreated control animals

From these results it was concluded that toluene simultaneously administered with benzene, may inhibit the metabolism of benzene in a competitive way

H van Riezen, *A neuromuscular preparation in vitro of the chick*

Department of Pharmacology, University of Utrecht and N V Organon, Oss

A neuromuscular preparation is made of the musculus tibialis anterior of the 2–10 days old chick It is bathed in Krebs Henseleit solution which is saturated at 37 °C with 95 % O_2 and 5 % CO_2 gas mixture The nerve is stimulated with pulses of 1 msec duration at 0 1 cps supramaximally

Tubocurarine and gallamine from 2 /ml decrease the twitch force registrated with Grass FT 03 However, 0 1 /ml suxa- or decamethonium give a slowly developing dose-dependent contracture on which the twitch is superimposed

From about 0 5 /ml the twitch height decreases during the increase of the contracture Small non blocking doses of curare-like compounds prevent the contracture, but potentiate the twitch decrease by methonium like drugs TEA increases the twitch height but does not give a contracture High doses of TEA block the effects of suxa and decamethonium on tension Atropine also blocks this contracture by methonium like drugs as does Hexamethonium

The preparation is very useful in differentiating neuromuscular blocking agents It shows both phases of the methonium like drugs,

1 *Photoelectric plethysmography* a pulsation measurement of the skin circulation
2 Venous occlusion plethysmography according to Whitney with mercury strands around the calf which gives an indication of the flow in the calf muscles

Vasodilating drugs were given intravenously in increasing doses up to 0 28 mg/min

RESULTS

1 For measurement of skin pulsations the big toe and the thumb are more sensitive than the skin of calf and forearm
2 In 5 healthy subjects an isoxsuprine derivate with a pure β sympathicomimetic action had a significant effect on the blood flow in the calf and no effect on the skin pulsations
3 In 5 patients with an atherosclerotic stop in the iliac of femoral artery this drug had no effect on the calf flow nor on the skin pulsations
4 In 6 healthy subjects isoxsuprine with a combined β mimetic and α lytic action had a significant increasing effect on the blood flow in calf and forearm and on the skin pulsations of the big toe and the thumb

CONCLUSIONS

1 With the described methods it is possible to investigate the influence of vasodilating drugs on skin and muscle haemo dynamics
2 The effect of these drugs on patients can differ from the effect on normal subjects

H van Rees *The influence of toluene on the metabolism of benzene in rats*

Dept of Pharmacology University of Leiden and Netherlands Institute for Preventive Medicine TNO Leiden

In industrial hygiene surveys the renal excretion of phenol is used as a criterion for the exposure to benzene As this exposure is often the result of benzene occurring as an impurity of toluene it was investigated whether simultaneous administration of the two substances would influence the metabolism of benzene As

were measured calorimetrically in two subjects doing uninterrupted constant work (7½ kcal/min) in a warm (35 °C) and dry (12 % RH) environment The subjects were not allowed to drink during the experiments The exposure time in the subsequent experiments was increased by 18 min up to a maximum of 144 min With a third subject who repeated the 72 min and 108 min experiments four times the accuracy of the measurement of the accumulated heat was assessed

Despite a continuous rise in rectal temperature owing to de hydration no increase in accumulated heat was found as long as the sweating rate remained at the required level The sweating rate remained at a constant level up to approximately 110 min After that it decreased and consequently the accumulated heat increased These findings support the concept of heat regulation rather than temperature regulation The misleading word body heat content should however, be avoided It is concluded that the regulatory model for thermal sweating includes not only body temperatures but also body mass The hypothesis is put forward that body water volume as relevant parameter of body mass, being itself a controlled magnitude influences the set point for thermoregulation or acts as the set point itself

D Spruit *Measurement of the water vapour loss of human skin in environmental air applying a thermal conductivity cell*

Department of Dermatology, R C University Nijmegen

In spite of its high sensitivity in detecting small changes of water vapour concentration in air, the thermal conductivity cell has not been applied widely in such measurements The most likely reason may be that it is well known that, theoretically, the indication of the instrument is not directly proportional to the concentration of the water vapour (GRUSZ and SCHMICK, 1928) However, in measuring the humidity of air, CHERRY (1965) found that the deviation from the direct proportionality is only small The sensitivity of his instrument should be twice as high measuring water vapour concentrations under 0 1 % as it should be at about 1–2 % The verification of the indication at a very low water vapour concentration gradient encounters many difficulties The difficulties were overcome and both direct and indirect verification were

depolarization, leading to a contracture of the slow fibres, and desensibilization, leading to decrease of twitch height.

Chr. L. Rümke and J. Noordhoek, *The influence of pretreatment with lynestrenol on the anticonvulsant effect of phenobarbitone and phenytoin in mice*

Department of Pharmacology, Free University, Amsterdam

In mice phenobarbitone (50 mg/kg s.c.) and phenytoin (50 mg/kg s.c.) exert an anticonvulsant effect in the supramaximal electroshock-test and in the chemoshock-test with bemegride, if administered 24 h previously.

These anticonvulsant effects of phenobarbitone and phenytoin are diminished if lynestrenol (4 mg/animal or.) is given, two days before the administration of one of these anticonvulsants. Lynestrenol alone (4 mg/animal or.), 2 or 3 days after its administration, was without effect in the electroshock-test or in the chemoshock-test.

The plasma level of phenytoin, 24 h after the administration of 50 mg/kg s.c., was 6.9 mcg/ml in the lynestrenol-pretreated mice, whereas it was 12.8 mcg/ml in controls The corresponding phenobarbitone levels (24 h after 50 mg/kg s.c) were 2.0 and 5.2 mcg/ml, respectively. After smaller lynestrenol doses (0.4 or 0 04 mg/animal), a similar effect on the plasma phenobarbitone level was observed.

Consequently, 48 h after pretreatment of mice with lynestrenol, the elimination rates of phenobarbitone and phenytoin from plasma are increased As phenytoin and phenobarbitone are mainly eliminated by metabolism in the liver, it is probable that lynestrenol accelerates the elimination of these drugs by enhancing the activity of the liver microsomal enzyme system

Whether this action of lynestrenol, which was observed in these animal experiments, should be taken into account in human pharmacotherapeutics, remains to be established.

J. W. Snellen, *The accumulation of heat during walking on the treadmill and its significance for the control of thermal sweating*

Dept. of Physiology, University of Nijmegen

Heat accumulations during exposures to work and heat load

the plasmas mixed with resp dextran fractions 100 000 to 140 000 and 47 000 to 67 000

2 In contrast to former trials, we were now able to separate the proteins of the sediments electrophoretically on paper The most important components observed were a fibrinogen peak and a smaller albumin peak The fibrinogen peak varies greatly according to concentrations and M W of the dextrans

3 In order to find out whether other proteins were present in the sediment, the supernatant from 1) was immuno-electrophoretically analysed according to Afonso The great variation in the planimetrically measured proteins of the controls showed that the areas, covered by each protein in the different dextran-plasma mixtures, did not fluctuate significantly

Still we believe that the constant relations observed prove that various globulins such as haemopexine, coeruloplasmine, fibrinogen, transferrine, IgM, IgA, are present, but probably not IgG Also lipoproteins and haptoglobine were found

4 This was confirmed by a similar analysis of the sediments, when an antiserum of a rabbit, immunized with the solved sediment of bovine plasma mixed with dextran B P, was used A definite relation between the quantity of the sedimented protein and the M W of the dextran and the percentage of its solution could not be established in these globulins

H J Th Thalen and Jw van den Berg, *The implantable R wave controlled pacemaker*

Laboratory of Medical Physics, University of Groningen

With electrical cardiac stimulation in cases of an A-V block in many patients a return of the sinus rhythm is found We observed this in 21 5 % of the pacemaker patients treated in Groningen The recovery of the sinus rhythm results in an interference between the sinus rhythm and the pacemaker rhythm causing an irregular heart action This is unpleasant for the patient and entails the danger of ventricular tachycardia or fibrillation when the pace maker impulse coincides with the vulnerable period of the heart-cycle To overcome these disadvantages the R-wave controlled pacemaker was developed

This pacemaker requires detection of the ventricular depolari-

realised Contrary to the results obtained by CHERRY, the indication of a GOW MAC microcell JDC 133 now showed directly proportional to the water vapour concentration, even at low concentrations under 0 1 %

The water vapour loss of 1 cm^2 forearm skin has been determined A flow of 10 ml environmental air/min has been directed through a conductivity cell, after that passed the skin and a second conductivity cell The accuracy of the measurements amounts to 0 01 mg water/cm^2 skin hour Carbon dioxide and other gases diffusing from the skin interfere only to about 5 % of the water vapour loss A measurement may be completed within two minutes if carried out applying environmental air, as it is not necessary to wait until the skin has become conditioned to changed circumstances Only after sweating some 15 min have to pass before the measurements can be started

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CHERRY, R H, in A WEXLER Humidity and Moisture Vol I, Reinhold, New York (1965) pp 539–551

W F H Stroer, *Electrophoretic observations on the sediments of dextrans and plasma proteins*

Pharmacological Dept of Povite Products N V, Amsterdam

It is a well known fact that some plasma proteins sediment when blood is mixed with dextran In order to analyse the composition of these sediments, the following experiments were performed

1 The supernatant obtained after centrifuging bovine haemolytic plasma, was mixed 4 to 1 with dextran fractions with a M W ranging from 47 000 to 518 000 × 10^{-3}, separated by electrophoresis and measured photometrically after staining with benzidine and afterwards with amidoblack It showed the greatest loss of fibrinogen in the 10 % solutions of the dextran fractions 83 000 to 153 000 This was confirmed in a similar experiment, when blood and dextran were mixed 1 1 In the 6 % and 10 % solutions the fibrinogen peak was smallest in

Our first R wave controlled pacemaker has been implanted in a patient in 1966 and functions well

J C de Valois *Regional cerebral blood flow*

Central Institute for Brain Research Amsterdam

The local clearance rate of an inert freely diffusible non metabo lized radioactive tracer measured over the cerebral cortex is an indicator of regional cerebral blood flow according to the Fick principle further elaborated by KETY (1951)

Suitable tracers for this determination are saline solutions of isotopes of Krypton or Xenon i e Kr^{85} and Xe^{133} To avoid extra cerebral isotope uptake and contamination of clearance curves a direct injection of the isotope into one of the internal carotid arteries or one of its main branches is preferable The clearance curves are then measured over the brain area to be studied and subsequently analysed The kind of radiation is important in regard to penetrative power of the radioactive particles and the kind of detector to be used Kr^{85} is almost exclusively a β-emitter (E_{max} = = 0 67 MeV) and Xe^{133} is a γ-emitter ($E_{max} \approx 0\ 081$ MeV) In the present series of experiments Kr^{85} was used From the maximal β energy of Kr^{85} a self absorption with a half thickness of 0 34 mm tissue can be determined This relatively small range makes Krypton very suitable for the measurement of regional *cortical* blood flow This however necessitates a craniotomy removal of dura and *suction* of blood and cerebro spinal fluid to avoid contamination of clearance curves

By *applying* this method to small animals (i e rabbits) one meets the difficulty of bridging the gap between the cerebral volume and the dimensions of the detectors commercially available In co operation with the Physics Laboratory of Philips Nederland N V small Lithium drifted Silicium p i n semiconductor detectors were developed The dimensions of this detector are 1 × 1 × 1 mm

During these experiments the *arterial pCO* was frequently measured with the Astrup micro apparatus and maintained between 35 and 40 mm Hg Neuroleptanalgesia was induced by Hypnorm[R]

Regional cerebral clearance curves obtained by this procedure and the calculated regional blood flows were in good agreement

zation. Research on patients and dogs showed that the amplitude of the depolarization wave decreases after the electrode-implantation and stabilizes after 4–6 weeks. The negative phase of the depolarization wave, after stabilization having an amplitude of ± 8 mV with intramural and ± 3.5 mV with endocardial electrodes, is used as trigger signal. Therefore the sensitivity of the detecting circuit is fixed at 1.9 mV for negative signals, with a frequency band of 25–150 Hz. The depolarization signal, obtained by means of a detecting electrode, is passed to the detecting circuit and, after modification and amplification, transmitted to a circuit designed to block the proper pacemaker. The pulse-forming circuit of the proper pacemaker is blocked if the heart frequency is higher than the fixed pacemaker frequency. The ventricles are stimulated by the pacemaker, however, if the heart frequency is lower than the pacemaker frequency. To prevent detection of the stimulation artefact, a second blocking circuit is started simultaneously with the stimulating impulse, blocking the detecting circuit for some time. After this blocking time the above-described cycle can start again.

The pacemaker has been tested in a number of animal experiments with good results. In the definite design improvements are made by using one electrode for detection and stimulation and by including a magnetic switch enabling analysis of the pacemaker, when blocked by the heart-activity.

The advantages of this pacemaker in regard to the continual asynchronous stimulating pacemaker are:

– no interference between pacemaker rhythm and heart rhythm
– lower energy consumption, since the energy consumption of the detecting and blocking circuits is less than the consumption of the stimulating circuit. The lifetime is therefore determined by the heart-activity itself and is roughly equal to the lifetime of the continual asynchronous pacemaker when blocked during 50 % of the time, becoming longer when the blocking percentage increases.

The advantages in regard to the P-wave triggered pacemaker are:

– simpler circuit
– more stable and greater trigger signal
– considerably lower energy consumption.

A Versprille *The effect of vagal stimulation on the S A and A V node of isolated young and neonatal mammalian hearts*

Laboratory of Clinical Physiology Department of Pediatrics University Hospital Leiden

DAWES *et al* (1957) described an inhibitory effect of N vagus on neonatal rabbit hearts However no information is available about the effect of vagal stimulation on hearts of young and neonatal animals with atrio ventricular rhythm

With the Langendorff perfusion apparatus the effect of vagal stimulation was studied on isolated hearts of neonatal (1 3 days old 3 experiments) and young rabbits (10–17 days old 9 exp) and young adult rats (5–6 weeks old 8 exp)

Atrio ventricular rhythm was established by cooling the sino auricular node A curved injection needle was placed against the region of the S A node and brought at low temperatures with cold water running through it In order to be sure of a selective cooling of the S A node the A V frequency has to be constant during cooling at different low temperatures In each experiment the discharge frequency of the A V node remained constant when cooling the S A node from 10–22 °C

The heart rhythm was measured by means of an electrocardiograph Two electrodes were placed on aorta and on apex of the heart respectively The A V rhythm was characterized by a negative P wave a shorter P Q interval and a lower frequency

Possible effects of changes in coronary perfusion on the S A as well as the A V rhythm occurring during vagal stimulation were controlled by stopping perfusion of the hearts for at least 10 sec In this time S A as well as A V frequency did not change significantly Therefore the percentage reduction of S A and A V frequency in the 7th–8th sec after beginning of vagal stimulation were compared

Stimulation of left as well as right vagus with frequencies of 4 8 and 16 Hz and maximal intensity has a clear inhibitory effect on S A frequency of all three groups of animals The A V frequency was proportionately more reduced about 1 5 times as much as the reduction of the S A rhythm

The experiments with the rabbit hearts did not show any significant differences between the stimulation-effect of right and left

with the values observed from the literature (LASSEN and INGVAR, 1963)

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A J Vergroesen, J de Boer and J J Gottenbos, *Effects of prostaglandins on perfused isolated rat hearts*

Biological Department, Unilever Research Laboratory, Vlaardingen

To elucidate the role of the heart in the cardiovascular reactions known to occur after the administration of several prostaglandins, isolated rat hearts were perfused according to Langendorff with different concentrations of a range of prostaglandins (PG) The criteria used were the contractile force of the myocard the frequency of the heart beat and the amount of fluid perfused through the coronary vascular system

PGE_1, PGE_2, PGE_1-217 (a dehydrated form of PGE_1) and $PGF_{1\alpha}$ and $PGF_{2\alpha}$ appeared to be active in concentrations of 0 01 µg/ml and higher The three PGE compounds acted mainly as vasodilators, PGE_1 being the most active and had little or no effect on the frequency of the heart beat The effect of PGE_1 on the contractile force was variable, mostly a moderate increase was observed, but sometimes a definite decrease occurred

On the other hand, the perfusion with $PGF_{1\alpha}$ or $PGF_{2\alpha}$ had practically no vasodilating effect but resulted in a sharp increase in mechanical activity of the myocard without causing a change in the frequency of the heart beat $PGF_{1\beta}$ and $PGF_{2\beta}$ seem to have hardly any activity in concentrations of 1 µg/ml and lower Several isomers and homologues of PGE_1 and of PGE_2 the homologues with 18, 19, 20, 21 and 22 carbon atoms were inactive in this experimental set-up

Some improvements of the system have to be mentioned

- the lifetime of a mercury tube is shorter the more a c is used, a higher-power tube on 70 % of its maximal output would lengthen the lifetime considerably,
- demodulation system will be improved by integrated circuits (i c 's),
- two monitor systems are necessary with a lower sensitivity than the receiver,
- photo-elements of a different sort will be used (fast photo-voltaic cells), placing of which is critical (phase relations),
- a second channel will be necessary for the amplitude parameter.

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ROHLÍCEK, V, Telestimulation by means of light transmission, World Medical Electronics 2, 254–256 (1966)

O L Wolthuis and E Meeter, *Spontaneous recovery of the neuromuscular transmission in the rat after intoxication with sublethal doses of cholinesterase inhibitors*

Medical Biological Laboratory of the National Defence Research Organization TNO, Rijswijk Z H

Groups of albino rats of about 200 g were intravenously injected with an LD_{50} of one of the following organophosphorous anti-cholinesterases DFP, soman, tabun, and S_{27}, S_{36} and S_{40}, sub stances, which are chemically related to systox The recovery of the neuromuscular transmission was studied by sacrificing one or more animals at appropriate t mes aft[illegible]

DER MEER and WOLTHUIS 1965)

With DFP and S_{27} no demonstrable recovery was observed during the first 4 days During the subsequent 10 days complete recovery took place With S_{36} and S_{40} partial recovery occurred in 3 days, with tabun this was already seen after one day Soman never evoked any impairment of neuromuscular transmission at this dosage

Sometimes haemorrhages were seen in the diaphragms 4 to 24 h after the anticholinesterase injection These rare haemorrhages

vagus In the rat hearts the right vagus showed a stronger inhibitory effect on the S-A as well as on the A-V node than did the left vagus These results with rat hearts are in agreement with those obtained by LEWIS (1914), who experimented with adult dogs

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P Visser, H van Ingen, A A Meyer and V Rohlíček,
Telestimulation by means of light transmission [1])

Department of Psychophysiology, Physiological Laboratory, University of Amsterdam

Telestimulation of animals is usually done by radiofrequency transmission For some years research has been done with cats continuously stimulated with sinusoidally modulated light The electrical responses of the visual cortex and their changes by distracting stimuli were studied Telestimulation of different cerebral sites was needed Many difficulties, such as detection of artificial potentials simultaneously with the biopotentials, can be easily overcome by using light transmission During light transmission, however, the average intensity of the light has to be constant *frequency modulation or pulse position modulation has to be used* The former was chosen, the demodulator being less complicated and less heavy Primarily fluorescent tubes have been used, but a carrier frequency of maximally 500 cs could be reached, which is too low in view of the FM modulation

The animal is free-moving and no direct "visibility" between receiver and light source can be guaranteed A superhigh mercury discharge tube (40 V, 1 4 A) showed the best results A current modulator with a carrier frequency of 14 kc/s is developed A very light demodulator and tuned miniaturized amplifier is attached to the back of the free moving cats (ROHLÍČEK, 1966) Square wave stimuli are transmitted of which one parameter only is to be transmitted *i e* width

1) The project was financially supported by the Netherlands Organization for Purely Scientific Research – Z W O – The Hague

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NETHERLANDS SOCIETY FOR PHYSIOLOGY AND PHARMACOLOGY

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were located at the end-plate region Inspection of diaphragms from DFP-rats with the low-power dissection microscope revealed a sequence of morphologic changes During the 3rd hour after DFP injection a "clearing" of the end-plate region may be visible, allowing more light to fall through this area From 3–24 h after DFP an increasing "greying" of the end plate region develops, which is also seen on the next day This "greying" appears to be an increase in optical density, and is accompanied by a general blurring of the contours of the muscle fibres and the nerve branches On the 3rd day after DFP a "clearing" of the end-plate region develops, which is bounded by grey areas on both sides This is still visible on the 4th day, whereas on the 5th day the muscle has regained a normal appearance

Soman, which does not interfere with end-plate function, produces no "greying" Reactivation of the cholinesterase in the rat with P_2S, one hour after DFP administration, prevents the development of "greying" Denervation of the diaphragm on one side just before DFP administration prevents "greying" from developing on that side Probably the abnormally high acetylcholine level at the end-plates either affects the muscle fibres directly or interferes with the local blood circulation These phenomena may slow down recovery during the first 4 days

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that quaternarization of certain α adrenergic blocking agents is well tolerated This in contrast to quaternarization of β adrenergic blocking agents which leads to a practically total loss of the β-adrenergic blocking activity The fact that quaternarization is allowed in the α adrenergic blocking agents indicates that the α-adrenergic receptors are located in such a way that they are easily accessible to the highly ionized quaternary compounds The fact that the quaternary derivatives of the β adrenergic blocking agents are practically inactive does not allow the conclusion that these receptors are hidden behind lipid membranes and therefore inaccessible to the quaternary onium compounds This since it may well be that the β adrenergic receptors do not tolerate quaternarization of these compounds The fact that also the tertiary amines, obtained by introduction of one methyl group in for instance the β adrenergic blocking agent Ko 592 results in a marked decrease (<0 005) of the blocking activity points in this direction

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E A Baarsma, P H Kylstra and A Ladan, *Depressor and pressor afferents in the vagal nerve of the rabbit*

Dept of Physiology, University of Utrecht

In a number of experiments it was shown that stimulation of the vagal nerve resulted in unexpected effects At the beginning of the stimulation period there was a sharp fall in blood pressure After some seconds this fall changed into a pressure rise to a higher level than the control level On cessation of the stimulus, a second fall could be recorded During this second decline, the pressure fell under the control level Just as during the first moments of the stimulation this fall in pressure changed into a rise

To analyse these effects, we performed twenty experiments In these experiments the central part of the cut vagal nerve was stimulated with rectangular current stimuli The strength of the stimuli was carefully selected The stimulation period lasted one

E J Ariens and A M Simonis, *Adrenergic blocking-agents Structure and activity*

Department of Pharmacology, University of Nymegen

A comparison of the α- and β adrenergic blocking agents with the adrenergic agents shows that the α adrenergic blocking agents have little or no chemical relationship, while the β-adrenergic blocking agents are very similar to the corresponding adrenergic agents, especially where the side chain is concerned (ARIËNS, 1967) For the α adrenergic blocking agents an interaction with accessory receptor areas – binding areas situated in the direct vicinity of, but differing from the α adrenergic receptors – is highly probable An analogous situation is reported for the antihistamines with respect to the histamine receptors, the antiserotonins with respect to the serotonin receptors and many of the anticholinergic compounds with respect to the cholinergic receptors (ARIËNS, 1966) The low specificity of various α-adrenergic blocking agents which have a relatively high antihistaminic and anticholinergic activity may be related to this mode of action The same holds true for the fact that many antihistamines and psychopharmaca such as the phenothiazine derivatives have an α adrenergic blocking activity, too The relations between the α adrenergic blocking activity (= 1) and the antihistaminic and anticholinergic activity respectively tested on the isolated vas deferens of the rat, on the isolated ileum of the guinea pig and on the isolated gut of the rat are piperoxan 1 – 0 16 – 0 11 Dibozane" 1 – 0 35 – 0 23, phentolamine 1 – 0 11 – 0 034 moxisylyt (' Opilon") 1 – 0 37 – 0 16 promethazine 1 – 500 – 5, chlorpromazine 1 – 3 17 – 0 13 thioridazine 1 – 0 31 – 0 063, thioproperazine 1 – 0 05 – 0 1 and prochlorperazine 1 – 0 63 – 0 8 For the β adrenergic activity a much higher degree of specificity is observed in this respect although some of these compounds *e g* propranolol have a weak antihistaminic activity The relations between the β adrenergic blocking activity (= 1) and the anti histaminic and anticholinergic activity respectively tested on the isolated tracheal muscle of the calf, on the isolated ileum of the guinea pig and on the isolated gut of the rat are propranolol 1 – 0 002 – <0 001, Ko 592 1 – <0 001 – <0 001, H 56/28 1 0 0014 – <0 001, MJ 1999 1 – <0 001 – <0 001

An interesting aspect of the *structure activity relationship* is

blood pressure When both groups are activated simultaneously, the first system, which is more rapid, causes the initial fall in blood pressure The second decline is caused by the cessation of the activity of the slower pressor system That the effects are less pronounced when the strongest stimuli are used, may be caused by the fact that small depressor active afferents come into action

We think some of our results are in accordance with those of DOUGLAS *et al* (1955) and with those of BONHOEFFER *et al* (1958)

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DOUGLAS, W W and W SCHAUMANN, J Physiol 132, 173–186 (1956)

D O E Gebhardt, *The influence of 5 fluorouracil and of aminopterin on limb regeneration of Ambystoma mexicanum A comparative study*

Rijksinstituut voor de Volksgezondheid, Utrecht

Published in Acta Physiol Pharmacol Neerl 15 (1969) 41

W A van de Grind, *A stochastic model of lateral inhibition in the human retina*

Department of Medical and Physiological Physics, University of Utrecht

Very extensive electrophysiological studies of the visual system of Limulus have been published by H K Hartline and his school These studies indicate that lateral inhibition is of utmost importance in the processing of visual information by the Limulus facet eye W Reichardt proved that in principle the mechanism of lateral inhibition is able to compensate for the blurring caused by the overlap of the visual fields of the ommatidia RATLIFF (1965) showed that extrapolation of these ideas to the human visual system leads to a good model of the Mach band phenomena

From the point of view of the quantum theory of vision it is of importance to note that the human retinal receptors are single-quantum-detectors This means that the signals from these receptors to the deeper structures of the retina have the character

minute. The duration of the rectangular stimuli was 5 milliseconds. Frequencies of 5, 10, 20, 40 and 100 stimuli per second were used.

RESULTS

In most experiments, the change in the blood-pressure depended on the stimulus-strength. Only in two experiments, the result of stimulation was always a fall in blood-pressure. In all other experiments mixed effects were obtained. The results can be rubricated as follows.

1. Effects obtained with threshold stimuli

These feeble stimuli always resulted in a decline in the blood-pressure. In most cases, the pressure-fall reached a constant level. On cessation of the stimulation period, the pressure rose to the original level. In most cases the pressure-fall was only small.

2. Effects obtained with stimuli of medium strength

If the stimuli were made slightly stronger, the blood-pressure declined to a minimum. This minimum appeared about ten seconds after the onset of the stimulus. After the minimum, the pressure started to rise slowly. The amount of this incline depended on the stimulus-strength With stronger stimuli, higher pressure levels were obtained. In most experiments, these levels remained constant throughout the stimulation period. Cessation of the stimulation was followed by a fast fall in the blood-pressure. This decline appeared to have the same time-constant as the decline at the beginning of the stimulation period

3. Effects obtained with strong stimuli

Stimuli of high intensity produced a rise in the blood-pressure in most experiments. After stimulation the pressure declined. Still higher intensities resulted in less pronounced effects. Sometimes the pressure even fell during stimulation.

CONCLUSION

We conclude that the mixed effects, described above, are caused by the simultaneous activation of at least three different groups of afferent fibres in the vagal nerve. The most sensitive afferents are pure depressor ones. Less sensitive afferents cause a rise in

between systematically varied loads, and physiological and psychological responses to these loads (HUETING, 1964, HUETING and SARPHATI, 1966)

The purpose of the present experiment was to test the validity of these results The power of the physiological and psychological variables to discriminate between non trained and trained subjects (Ss_n and Ss_t) was investigated

METHOD

The Ss performed work on a bicycle ergometer The load was increased, for the Ss_n by 10 Watts every minute, for the Ss_t by 30 Watts every 3 min This discrepancy between programs had no consequence for the present purpose

The physiological variables used were both heart frequency (beats/min), and respiratory minute volume (ml/min) together with oxygen consumption (ml/min), which were measured in a closed-system spirograph

As a psychological index the load perceived by the Ss was indicated on a rating scale, during exercise the Ss matched the angle of the pointer of a voltmeter with the perception of the effort by moving the pointer upwards The corresponding voltages were recorded

The Ss were males between 19 and 25 years old The Ss_n, 14 normal healthy students, performed the exercise till their heart frequency reached 170 The Ss_t, 11 top amateur racing cyclists, performed till exhaustion, indicated by themselves as 90° on the voltmeter These Ss repeated the experiment after a 2 week interval

RESULTS

All Ss_n attained a level of 190 Watts, all Ss_t 330 Watts Within these ranges regression equations were calculated directly from the data (method of least squares), for the Y's as the dependent variables, and X as the independent variable in minutes 1, 2, 3 etc

For Ss_n and Ss_t the regression equations were respectively heart frequency $Y = 4\,6X + 79$ and $Y = 2\,4X + 76$, respiratory minute volume $Y = 2\,400X + 5\,400$ and $Y = 1\,700X + 12\,500$, oxygen con-

of a Poisson process Another important result of psycho physical studies of human vision is that even after complete dark adaptation at least 2–12 quanta have to be absorbed within a restricted area and time interval for perception to take place

Under these circumstances the deeper retinal cells apparently function as "coincidence detectors" In view of these facts an important question is how coincidence detectors can inhibit each other in a manner similar to the non stochastic lateral inhibition in the eye of Limulus A coincidence gate (C gate) can be defined as a multi input threshold gate where every incoming pulse is 'remembered' T time units (T is the coincidence interval) A C gate with 2 inputs with weights $+x$ resp $-x$ and a threshold $+x$ performs briefly stated as follows

The C gate gives an output in response to a pulse on the excitative ($+x$) input unless a pulse arrived at the inhibitive input less then T time units before

This simple mechanism proved to be an excellent stochastic counter part of the non stochastic type of inhibition and consequently Mach band phenomena can be modelled in this way

The described mechanism is slightly different from the one treated mathematically by TEN HOOPEN (1966) in that the inhibitive pulses in our case have a time restricted influence When $1/T$ is small compared to the average input frequencies our model converges to the one described by Ten Hoopen

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J E Hueting A M Coffeng M van Leeuwen and A J Poulus *A comparison of physiological and psychological responses during physical exercise in non trained and trained male subjects*

Physiological Laboratory and Coronel Laboratory for Environmental Hygiene University of Amsterdam

In previous experiments significant intercorrelations were found

nerves on cardiac arrhythmia but considered it more disturbing than interesting

In our laboratory, a marked influence of vagal stimulation on the time interval relation of the isolated rabbit heart was found Retrogradly perfused rabbit hearts were used and the R R intervals of the surface electrogram were measured (for further methods see GERLINGS (1966) When the vagal nerves were not stimulated the intervals showed an amazingly constant time interval relation (deviations of appr 4 msec) Supraliminal stimulation of the vagal nerves with frequencies of 5 7½, 10, 14 18 and 22 Hz, however, showed an increasing inconstancy in the time interval relations No difference was seen between stimulation of the right or the left vagal nerve

Since heart rate dropped considerably during vagal stimulation the augmented variability in time interval relation could possibly be an effect of the lowered frequency To eliminate this effect the sympathetic nerves of an isolated heart showing a constant time interval relation were stimulated, resulting in a 50 % increase of the heart rate Still no difference in time interval relation could be found The vagal nerves were then stimulated (appr 15 Hz) lowering the heart rate again to the original level Now a distinct increase in the irregularity of the time interval relation was found

From these experiments we conclude that the vagal nerves have an influence on the time interval relation of the heart The sympathetic nerves do not have such an influence

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sumption $Y = 123X + 1\,060$ and $Y = 107X + 971$, effort intensity $Y = 4.4X - 4$ and $Y = 3.0X - 40$

In all these cases the coefficient is smaller for the Ss_t than for the Ss_n, hence the slopes of the lines for the Ss_n are steeper Moreover, for Ss_t the regression line for effort shows a considerably lower level, which lends still more power to this measure

Eliminating X from the equations gives the following relationship between the two groups for heart frequency $HF_t = 0.5HF_n + 35$, for effort intensity $EI_t = 0.7EI_n - 37$ Extrapolating the regression line of the last equation suggests a wide range of discrimination

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S. G T Hulst and D de Wied, *Changes in body temperature and water intake following intracerebral implantation of carbachol in rats*

Department of Pharmacology, Medical Faculty, University of Utrecht, Vondellaan 6, Utrecht

To be published in Physiology and Behavior (1967)

G Jambroes, E D Gerlings and A G Willebrand, *The influence of the vagal nerves on cardiac time-interval relations*

Department of Physiology, University of Utrecht

The ECG of trained athletes at rest revealed a considerable inconsistency in the time-interval relation of the R-R intervals

This same inconsistency in time interval relation was found in experiments with isolated rabbit hearts when the vagal nerves were stimulated

It seemed useful to find out to what extent the vagal nerve tone was a crucial point in the generation of this phenomenon

In literature the sinus arrhythmia is generally considered to be a respiratory phenomenon DE GEEST *et al* (1965), who carried out investigations in this field, found an influence of the vagal

From these findings it is concluded that also during random stimulation the contractile parameters of an isolated heart are closely related to the preceding RR intervals It is conceivable that this relation is a contributing factor in the variability of hemodynamic parameters found during atrial fibrillation in men

T PIEK, *Action of the venom of the wasp Microbracon hebetor Say on the somatic neuromuscular transmission of the moth Philosamia cynthia Hübn*

Pharmacological Laboratory, University of Amsterdam

The contents of the venom bladder of *Microbracon* which are about 0 009 µl (HASE 1924), can paralyse more than 50 pounds of larvae of the wax moth (BEARD, 1952) The venom has no effect on either the efferent or the afferent nerves or the activity of the visceral muscles (BEARD, 1952, PIEK, 1966)

Field stimulation of paralysed moth larvae results in a con traction of the integumental muscles At intracellular stimulation the flight muscles of *Philosamia* show active responses not affected by *Microbracon* venom Neither does the venom affect the effective membrane resistance of muscle fibres

Intracellular records of membrane potentials in flight muscle fibres of *Philosamia* show miniature excitatory postsynaptic potential changes (MEPSP's) with a frequency varying from 50–250 potentials per min in the different fibres The average amplitudes of the MEPSP's vary from 30–50 µV in the different fibres

The most marked effect of *Microbracon* venom is on the frequency of the MEPSP s After an initial increase the frequency gradually decreases to zero while the amplitude is decreased by at most 30 % This indicates a presynaptic effect of the venom

At a relatively high initial frequency the time-course of the changes in frequency is faster than at a relatively low initial frequency

In addition it is shown that after administration of *Microbracon* venom the time-course of the paralysis of the flight muscles is faster in muscles stimulated indirectly once per second than in muscles stimulated indirectly once per minute

F L Meijler, J Strackee, F L van Capelle and J C du Perron Maas, *Computer analysis of the interval contractility relationship during random stimulation of the isolated heart* [1])

Department of Cardiology and Clinical Physiology, Wilhelmina Gasthuis, University of Amsterdam, Laboratory of Medical Physics, University of Amsterdam

Atrial fibrillation is a common rhythm disturbance of the heart in men During this type of arrhythmia the heart contracts very irregularly and it can be seen that in general beats differ from the preceding one and from the next

For the understanding of the hemodynamics of patients with atrial fibrillation, some knowledge of the relationship between the duration of RR interval and the contractile behaviour of the myocardium may be of importance However, this relationship has not yet been studied in hearts contracting irregularly For such a study the aid of a computer is almost essential

First the behaviour of RR intervals of patients with atrial fibrillation was studied After suitable conversion of the E K G available on magnetic tape a computer (IBM 7094) produced the serial auto correlation function and the histogram of approximately 2500 RR intervals It was found that the intervals were random and the histograms rather skew Next we produced random rhythms with the aid of a radioactive source and a Geiger Müller counter By scaling the impulses and introducing a dead time histograms matching those of the patients were obtained These rhythms were then used to stimulate isolated perfused rat hearts The E K G and the isotonic contractions were digitized and processed in the computer Serial cross correlation functions were computed between the RR interval on one hand and contraction-height area and maximum of the first derivative of the contraction on the other hand

The first order cross correlation coefficient between RR interval and contraction height turned out to be 0 6, between RR interval and contraction area 0 8 and between RR interval and maximum of first derivative 0 5 Although small the second order coefficient was definitely negative in all instances

[1]) Supported by the Organization of Pure Research (Z W O) s Gravenhage The Netherlands

L H van der Tweel, F van Capelle and F L Meijler,

Synchronization of pacemaker cells

Laboratory of Medical Physics, University of Amsterdam, Department of Cardiology and Clinical Physiology, Wilhelmina Gasthuis, University of Amsterdam

As early as 1928 Van der Poll compared the electrical activity of the heart with relaxation oscillation Using this as a working hypothesis he was able to simulate the E K G in a model The later development of micro-electrode techniques indeed demonstrated the feasibility of this concept for pacemaker cell activity

Since artificial electrical stimulation has up till now always been effected by means of pulses of short duration resulting in an all-or nothing response, it was thought of interest to study whether certain characteristics regarding synchronization of a relaxation oscillator are present in pacemaker tissue in isolated perfused rat hearts

For this purpose a sinusoidal current was applied to the bath in which the heart was submerged, by means of two electrodes It was found that, in a restricted frequency range around the spontaneous rate of the heart (in our setup 4–5 c/sec), the complexes fell at a certain site in the sinusoidal cycle, depending on current strength and frequency

As in technical relaxation oscillators this synchronization was also found for frequencies of the synchronizing current, being multiples of the spontaneous rate

Right or left auricular bipolar complexes did not change when current was applied as long as this did not exceed a limiting value In the experiments in which the ventricular R wave could be identified in the auricular leads, the P-R time was found to be equal for the spontaneously beating and the synchronized heart

With increasing current strength the *form* of the complexes changed more or less suddenly, while at the same time the frequency selectivity of the phenomenon disappeared Moreover the complexes occurred at another place in the sinusoidal cycle

After removal of the auricles the described phenomena could be demonstrated on a ventricular level at much lower rates

As far as this macro-electrode technique permits, it seems justified to conclude that the heart can be synchronized in a way,

These results indicate a block of the synthesis of the transmitter substance, rather than a block of transmitter release

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PIEK, T, Insect Physiol 12, 561–568 (1966)

C Romijn and W Lokhorst, *The respiratory metabolism of the hen's embryo at a low oxygen pressure*

Laboratory for Veterinary Physiology, University of Utrecht

The heat production and heat dissipation of incubated eggs have been determined under simulated "high altitude conditions", as well as in air with a decreased oxygen content Under both conditions the energy metabolism of the embryo is highly correlated with the pO_2 in the incubating air A low oxygen pressure results in a decrease of the respiratory metabolism and in a slightly retarded hatching of the chicken

Low oxygen content at normal atmospheric pressure has a much more pronounced effect than the "high altitude" condition Moreover, the heat elimination in the former condition is greater than the heat production

This phenomenon may be explained by a smaller evaporation at normal atmospheric conditions The water loss from the eggs is within wide limits independent of air velocity but in linear correlation with the water vapour tension in the surrounding air and increases with the lowering of the total pressure of the incubating air

The limiting factor in oxygen supply to the embryo is therefore the water content of shell pores and membranes

At conditions of "high altitude" the desiccation of the pores in the egg shell may be considered as an adaptation to the unfavourable conditions in embryonic development

not only of the oxygen concentrations, but also of the volume expired, in the carbon dioxide free gas

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WEIR, J B DE V, J Physiol 109, 1 (1949)

D de Wied, *The effect of ACTH and ACTH fragments on avoidance learning*

Department of Pharmacology, Medical Faculty, University of Utrecht, Vondellaan 6, Utrecht

Removal of the pituitary gland or the adenohypophysis only, markedly diminishes the ability of rats to acquire an avoidance response (APPLEZWEIG and BAUDRY, 1955, DE WIED, 1964) The administration of a long acting ACTH preparation or a hormone substitution therapy with cortisone, testosterone and thyroxine improves the learning ability of adenohypophysectomized rats (DE WIED, 1964)

The inability of the adenohypophysectomized rat to acquire an avoidance response may be attributable to a general debilitation of the animal owing to lack of adequate hormonal supply It may also be due to the absence of ACTH This was determined with the aid of ACTH fragments which do not exhibit noticeable corticotrophic effects

Hypophysectomized male rats were trained in a shuttle box The CS was a buzzer presented for 5 sec followed by the US of shock if the animal did not make a barrier crossing Ten conditioning trials were administered each day for 14 days Animals were treated with 2 dose levels of synthetic ACTH β 1–24, α MSH or ACTH 4–10 Zn phosphate complexes of these peptides were prepared (DE WIED, 1966) and injected subcutaneously every other day during acquisition The treatment was started one day prior to the first conditioning trial Sham-operated rats served as controls

It appeared that α MSH and ACTH 4–10 markedly improved avoidance learning in a manner quite similar to that observed when ACTH β 1–24 was used in a dose of 20 μg Dose response curves of these 3 peptides were nearly identical

Since ACTH 4–10 is devoid of corticotrophic activity, the results indicate that the behavioral effect of ACTH in hypophysectomized

fundamentally differing from the conventional stimulation with pulses

B F Visser, *Determination of oxygen uptake without carbon dioxide measurement*

Pulmonary Function Laboratory, University Hospital of Utrecht

The method of WEIR (1949) for the calculation of metabolism (K') from oxygen concentrations in inspired (O_i) and expired air (O_e)

(17) $$K' = (O_i - O_e)0\ 0504 \qquad (1)$$

is based on the coincidence that the calorie value of one litre of oxygen (K) as a function of the respiratory quotient (R)

(9) $$K = 3\ 9 + 1\ 1R \qquad (2)$$

almost equals 5 % of the correction factor to be applied because of the volume difference between inspired and expired air

$$79\ 07 + 20\ 93R \qquad (3)$$

It is impossible, however, to calculate the oxygen consumption in this way without knowing R The following reasoning presents a solution to this problem

If there existed beings not producing carbon dioxide at all, living in a carbon dioxide-free atmosphere, their oxygen con sumption (O) could be calculated from the volumes inspired (V_i) and expired (V_e) and the corresponding oxygen concentrations O i and O e

$$O' = V_i \cdot O'_i - V_e\ O'_e \qquad (4)$$

In this case

$$O = V_i - V_e \qquad (5)$$

Substitution of V_i from Eq (5) into Eq (4) and solving for O' yields

$$O'/V_e = (O'_i - O'_e)/(1 - O_i) \qquad (6)$$

The condition for validity of Eq (6) can be simulated by first absorbing carbon dioxide and then performing the measurements,

The results were compared with those obtained by similar experiments with rat liver preparations as described by AL-KASSAB *et al* (1963), BOOTH *et al* (1961) and COHEN *et al* (1964)

The possibilities of comparative biochemistry of foreign compounds for comparative toxicology were discussed

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COHEN, A J J N SMITH and H TURBERT, Biochem. J 90, 457 (1964)

O L Wolthuis J F Anthoni and W F Stevens, *Inter-animal transfer of information by brain extracts*

Medical Biological Laboratory of the National Defence Research Organization TNO, Rijswijk (Z H)

In a modified T maze rats were trained twice daily to avoid foot shock by escaping into the lighted alley After 15–16 days, when 80 % or more of the responses were correct, the animals were decapitated their brains homogenized and the homogenate centrifuged

Naive recipients were injected i p with the resulting supernatant, each animal receiving a fixed dose equivalent to approximately two thirds of a donor brain Control recipients received the same dose of identically prepared supernatant from the brains of untrained donors

Four series of 10 experimental versus 10 control recipient rats were trained in the same way as the donor animals, the combined results show that learning speed in the recipients injected with trained supernatant is significantly enhanced ($p_2 = 0.006$)

To investigate whether a non-specific stimulating effect could enhance learning in the T maze used the effects of strychnine, pervitin and Mg Pemoline on speed of learning were investigated In the dosages used none of these substances enhanced learning

rats is extra-adrenal Studies on other parameters such as escape and exploratory behavior as well as on the general condition of the hypophysectomized animal should be carried out to determine the specificity of this heptapeptide on avoidance learning

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J G WIT and P LEEUWANGH, *Aspects of mercapturic acid formation in birds*

Institute of Veterinary Pharmacology and Toxicology, Biltstraat 172, Utrecht

The ability of birds to form mercapturic acids (N-acetylcysteine derivatives of foreign compounds) is relatively unknown In mammals, the initial stage in the biosynthesis of mercapturic acids is the reaction of the foreign compound with glutathione, occurring in the liver The glutathione derivative can be degraded by enzyme reactions to give a S cysteine derivative, which is N-acetylated enzymatically to yield a mercapturic acid (see BOYLAND and BOOTH, 1962)

The metabolic pattern of 1,2 dichloro 4 nitrobenzene and 2,3,5,6-tetrachloronitrobenzene in birds (pigeons) was investigated in comparison with rabbits (BRAY *et al*, 1957, BRAY *et al*, 1953) It was found that pigeons can form mercapturic acids with both compounds Pigeons convert part of 1,2 dichloro-4 nitrobenzene to 3,4,3',4' tetrachloro azoxy benzene and 3,4-dichloro aniline, as is the case with rabbits Pigeons, however, are unable to metabolize 2,3,5,6 tetrachloro nitrobenzene to 2,3,5,6 tetrachloro aniline and 2,3,5,6 tetrachloro-4 amino phenol, found as metabolites in rabbit urine Experiments *in vitro*, using the high-speed supernatant of pigeon liver, demonstrated the presence of glutathione-S aryl-transferase, as indicated by the enhanced liberation of nitrite-ions from 4-nitropyridine N-oxide and 2,3,5,6 *tetrachloronitrobenzene* and by the formation of S (2 chloro-4 nitrophenyl)-glutathione and S-(2,4-dinitrophenyl)-glutathione from 1,2-dichloro-4-nitrobenzene and 2,4-dinitrochlorobenzene respectively

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NETHERLANDS SOCIETY FOR PHYSIOLOGY AND PHARMACOLOGY

PROCEEDINGS 107TH MEETING, AMSTERDAM JANUARY 13, 1968

These results are in accordance with recent findings described in the references

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e) Presence of precursors
f) Specific release mechanisms
g) Identical action
h) Pharmacological identity

(*a, b, c, d*) All components of the cholinergic system are present in the central nervous system, but not in the peripheral nerves of insects The contents of free and bound acetylcholine are greater than is known of vertebrates, the same holds for cholinacetylase and cholinesterase (COLHOUN, 1958) These components have been proved to be present by chemical and histochemical methods An interesting observation is the rather high cholinesterase activity in the nerve sheath that can form an efficient "biochemical barrier" (TREHERNE)

(*h g*) The action of acetylcholine of insect preparations was studied by many authors (*e g* PROSSER, 1940, ROEDER, 1948, 1950, KERKUT, 1965) The differences in materials, methods and results raised controversy concerning the role of acetylcholine Other transmitters, well known from vertebrates, have been identified in nervous tissues of insects, however, their actions still remain obscure Remarkable is the importance of cholinesterase inhibitors, many of which are used as insecticides

Amino acids have received considerable attention, especially in the last few years Several authors assume L-glutamate to be an exciting transmitter and GABA an inhibiting one in insect neuro muscular synapses The author of this abstract could not confirm these observations (FLOREY, 1967) preferred to use the term "*modulator substance*" for compounds (*e g* amino acids) that affect the performance of the nervous system

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P N Aarsen, *Sensitization of guinea pig ileum to the action of bradykinin by trypsin hydrolysate of ox and rabbit plasma*

Pharmacological Laboratory, University of Amsterdam

Accepted for publication by Br J Pharmac Chemother

G Becht, *Transmitters in the nervous system of insects*

Department of Zoology, University of Nijmegen

The nervous system of arthropods generally contains a dorsal brain ganglion and a pair of longitudinal ventral nerve cords with segmental ganglia arranged in a ladder-like configuration, peripheral nerves connect them with several organs Depending on the shape of the species, there are many variations on this pattern

The anatomical structure of the ganglia is unlike those of vertebrates *A superficial connective tissue sheath is presumably equivalent* to the vertebrate perineurium, in the insects the non cellular portion will be referred to as "neural lamella", the cellular one as "perineurium" The next peripheral layer contains nerve cell bodies, which are interconnected in the central part of the ganglions by a meshwork of axons, the neuropile, Schwann- and glia cells and many extracellular spaces are situated near the periphery of the ganglion Important achievements in the knowledge of structure and function are described and summarized by TREHERNE (1965)

Central synaptic transmission is essentially similar to that of vertebrates However neuromuscular junctions of arthropods differ from those in vertebrates in that only a limited number of neurones may innervate each muscle as well as each muscle fibre This so called polyneuronal innervation is connected multiterminally with muscle fibre Both kinds of synapses the neuromuscular and central ones contain numerous synaptic vesicles localized at the *presynaptic membranes*

Several authors developed criteria for the identification of transmitters (COLHOUN 1963 FLOREY 1967 HEBB and KRNJEVIĆ, 1962 WERMAN 1966) They can be listed as

a) Presence of inactivating enzyme
b) Presence of transmitter
c) Collectability of transmitter
d) Presence of synthetizing enzyme

change in direction of activation of the S A node may cause a change in the configuration of the transmembrane action potential of the nodal fibres

An attempt was made to measure this change more precisely In the isolated rabbit atrium action potentials of fibres in the S A node were measured with conventional glass micro-electrodes When a pacemaker cell was impaled the atrium was electrically driven by means of a bipolar electrode on the right auricular appendage when the driving had been maintained for 10 sec the driving was continued with a bipolar electrode on the right atrial posterior wall This alternation in direction of activation was repeated every 10 sec as long as the micro-electrode remained in the fibre Data from an impalement were used for calculation when we had been able to pace the preparation twice from either electrode The driving frequency was kept at 10 % above the spontaneous frequency at the start of the experiment

The action potentials were stored on magnetic tape After the experiment we measured the amplitude the maximal rate of depolarization and the duration of the action potential on 1/4 and 3/4 of the amplitude Per 10 seconds period of driving the mean values of these parameters were calculated from 20 subsequent action potentials and their standard errors This was done for 15 cells taken from 4 experiments The results can be summarized as follows

1 In all cases in which 4 parameters could be measured at least 2 parameters changed significantly according to the Student s T test ($p = 0\ 01$) when the activation pattern was changed
2 It was impossible to classify the change in terms of fibre type or localization the parameters seem to change independently of each other
3 Of the measured parameters the maximal rate of depolarization is affected most by a change of the site of stimulation An increase or decrease of more than 20 % was frequently observed In most cases the change of the other parameters was less than 10 %

F I M Bonke and L N Bouman, *Disturbances of S A node function as caused by atrial premature beats*

Department of Physiology, University of Amsterdam

In a previous paper BONKE *et al* (1968) we described the relation between the length of the atrial cycle before and after the premature beat Impulse conduction in the isolated, spontaneously beating right atrium of the rabbit was measured with micro electrodes The earlier in the atrial cycle a single electrical stimulus was applied to the atrium, the lower was the conduction velocity of the impulse By recording simultaneously with two bipolar electrodes on the surface of the atrium and one micro electrode in the S A node, we have obtained evidence that after a discharge by a premature beat a shift of the pacemaker occurred within the node

The slope of the diastolic depolarization as recorded from the fibres of the S-A node was found to be depressed after a premature beat The depression was more pronounced in true pacemaker fibres than in latent ones We assume that this unequal depression and the very slow conduction of the impulse through the S A node cause the shift of the pacemaker

In our opinion two factors might explain the increased length of the premature beat cycle

1 the decrease of the conduction velocity in the atrium and the S-A node when the premature beat is elicited at an early moment in the atrial cycle
2 the shift of the pacemaker within the S A node

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BONKE F I M, L N BOUMAN and H E VAN RIJN Acta Physiol Pharmacol Neerl (1968) in the press

L N Bouman F I M Bonke and H E van Rijn, *Directional influences on the action potential in the S-A node of the rabbit*

Department of Physiology, University of Amsterdam

From experiments on the conduction velocity of impulses through the S A node of the rabbit, we obtained evidence that a

showing an ED_{50} between 100 and 1000 ng of venom gland extract per 100 mg body weight All other insects tested did not react to a venom dose up to 1000 ng per 100 mg body weight

D B Faber, *The pharmacology of polypeptides enhancing the action of bradykinin*

Pharmacological Laboratory, University of Amsterdam

OSBAHR *et al* (1964) showed that human fibrinopeptide A enhances the effect of bradykinin on the rat uterus A substance increasing the effect of bradykinin on the guinea pig ileum was shown by AARSEN (1968) to be present in ox and rabbit plasma treated with trypsin Highly purified human fibrinogen (96–100 %) was treated with bovine thrombin Human fibrinopeptide A was isolated using Sephadex filtration followed by column electrophoresis on Sephadex With the same separation methods, a negatively charged peptide was isolated from rabbit plasma treated with trypsin

The effect of fibrinopeptide A was studied in the guinea pig ileum, rat uterus, rat vas deferens, rabbit ileum, guinea pig bronchi, guinea pig lung vessels, rat aorta and sheep coronary artery In *all these* preparations the effect of bradykinin was enhanced, whereas eventual effects of histamine, acetylcholine, *serotonine*, angiotensin, eledoisin, oxytocin, ergometrin adrenalin and noradrenalin were not enhanced Both fibrinopeptide-A and the rabbit plasma peptide increase the spontaneous contractions *of the rat* uterus (in oestrus), and show an inhibition of the spontaneous contractions of the rabbit ileum followed by an increase in *tone* These eff[illegible]
E[illegible]
I[illegible] higher than that of bradykinin *Apart from* striking pharmacological similarities, the two peptides *are also* similar with respect to molecular weight, iso-electric point and absorption spectrum and therefore they may be *identical* Since some of their effects resemble those of bradykinin, these peptides may have the same site of action as bradykinin

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D. Drenth, *Some aspects of the collection and the action of the venom of Microbracon hebetor Say*

Pharmacological Laboratory, University of Amsterdam

Venom preparations were obtained from female *Microbracon* wasps in the following ways

1 extraction of whole wasps
2 extraction of isolated venom glands
3 electrical stimulation of large numbers of wasps simultaneously
4 stimulation with ether vapour of large numbers simultaneously

In order to compare these preparations as to yield activity and purity, the following investigations were performed

1 determination of dry weight after freeze drying
2 determination of the ED_{50} 24 h after injection into larvae of *Galleria mellonella* L
3 determination of the protein content
4 disc electrophoresis

The purest preparations were obtained after electrical stimulation but the yield is too low to make this method suitable for practical use Both extraction of venom glands and stimulation with ether vapour give high yields of very active preparations without marked differences in purity Since mass stimulation with ether is much simpler than the preparation of venom gland extracts ether stimulation is chosen as the routine method for the collection of *Microbracon* venom In diluted solutions *Microbracon* venom rapidly loses its biological activity The stability cannot be improved by the addition of salts but bovine serum albumin and some other proteins have a stabilizing effect The optimum stability lies between *p*H 8 and 9 Concentrated venom solutions and freeze dried preparations can be stored at -20 °C without apparent loss of activity

Representatives of different insect orders were injected with increasing doses of a venom gland extract A high sensitivity for *Microbracon* venom seems to be restricted to the order of the *Lepidoptera* Most members of this order show an ED_{50} smaller than 10 mg of venom gland extract per 100 mg body weight From other insect orders only *Musca domestica* L (*Diptera*) and *Perillus bioculatus* Fab (*Hemiptera*) are susceptible to the venom

into the impaled cell is electrotonically conducted to the pacemaker cells of the preparation

In the atrium preparation, however, so much current is dissipated that the pacemaker region is not at all influenced by it Current dissipation might be caused by low intercellular resistance in combination with the large diameter of the preparation.

In the small cluster there will not be so much current dissipation In some cases current of sufficient intensity will reach the pacemaker region and alter its discharge frequency by changing the membrane potential and the velocity of the diastolic depolarization

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SPERELAKIS N and D LEHMKUHL, J Gen Physiol 47, 895 (1954)
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C van der Meer, *General considerations on an investigation of paralysing wasp venoms*

Pharmacological Laboratory, University of Amsterdam

The purpose of our investigation is the elucidation of the chemical structure and the mechanism of action of paralysing wasp venoms The following phases in this investigation can be distinguished

SELECTION OF THE WASPS

The selection of species that can be obtained in large numbers meets with difficulties, since very little is known about the biology of these wasps as well as about methods of cultivation.

The venoms of different species appear to differ in mechanism of action and chemical composition

COLLECTION OF THE VENOM

Since the collection of the natural venom meets with difficulties, methods for artificial collection are being used

BIOLOGICAL STANDARDIZATION

Biological units must be defined as a measure of the activity of the venoms These units may vary considerably, *e g* owing to changes in condition of the test animals (insects)

BAUER, G., E. ZIEGLER und H. KONZETT, Naunyn Schmiedebergs Arch Pharmak Exp Path 254, 235 (1966).
OSBAHR, A. J., J. A. GLADNER and K. LAKI, Biochim Biophys Acta 86, 535 (1964).

H. J. Jongsma, R. J. v. d. Stadt, C. Borst and L. N. Bouman, *The effect of polarizing current on the automaticity of isolated heart muscle preparation*

Department of Physiology, University of Amsterdam

The mechanism of impulse conduction in the heart is still the subject of controversy. Both an ephaptic and a synaptic mechanism are advocated (SPERELAKIS and LEHMKUHL, 1954, WOODBURY and CRILL, 1961). We have re-investigated this problem by using

a. isolated atrium preparations including the sino-atrial node (diameter 2–3 cm),
b. clusters of atrial cells cultured *in vitro* (diameter 100–300 micron).

In both cases a bridge circuit (ARAKI and OTANI, 1955) was used for the simultaneous application of current and recording of action potentials with one micro electrode.

*Application of hyperpolarizing or depolarizing currents of maxi*mally 70 nA to the atrium preparation had neither an effect on the discharge frequency of an impaled fibre nor on the frequency of the whole preparation as measured with a surface electrogram.

Application of currents of maximally 7 nA to cell clusters mostly had no effect, sometimes a small effect and a few times a pronounced effect. In the latter cases a depolarizing current increased the discharge frequency of the impaled cell, whereas a hyperpolarizing current decreased and sometimes even completely abolished the spontaneous electrical activity. The magnitude of the effect depended on the intensity of the current applied, on the diameter of the clusters and on the initial contraction rate of the clusters, which was measured with a photo diode. The contraction frequency of the whole cluster always remained synchronous with the discharge frequency of the impaled cell, even when it obviously was a latent pacemaker cell. In our opinion these results favour the hypothesis of ephaptic impulse conduction. The current injected

cy is by far the more important In *Philosamia* however, the amplitude of the MEPSP s is not affected, but the frequency of the potentials shows an initial increase followed by a decrease, similar to the effect of *Microbracon* venom in this species (PIEK, 1969)

In both species studied *Philanthus* venom mainly affects the frequency of the MEPSP s rather than the amplitude and therefore may be assumed to act predominantly on a presynaptic site

After administration of *Philanthus* venom, the onset of paralysis of the muscle is speeded up by activity of the neuromuscular system by stimulation of the nerve This may be an indication that the venom affects either the synthesis or the storage mechanism of the neuromuscular transmitter substance

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PIEK, T, Acta Physiol Pharmacol Neerl 15 (1969) 87

R T Simon Thomas, *The collection and breeding of wasps producing a paralysing venom*

Pharmacological Laboratory, University of Amsterdam

For an investigation on the mode of action and chemical structure of paralysing wasp venoms large numbers of female wasps must be available, preferably throughout the year Large numbers may either be reared in the laboratory or collected in the field

Parasitic wasps that are suitable for mass culture must fulfil the following requirements 1 mating in a small space, 2 no diapause, 3 large reproduction, 4 easy mass culture of the host

Microbracon hebetor Say (Braconidae), which uses small moth larvae as its host, fulfils all these requirements One female produces an average offspring of about 12 females At present our colony produces 400–500 females daily The following requirements can be formulated for predatory wasps that may be collected in the field 1 easy identification as to species and sex, 2 occurrence in dense populations, 3 good survival during transport.

Philanthus triangulum F (Sphecidae) meets these requirements Unfortunately this species has disappeared in Holland, probably following a series of six cool summers from 1961 to 1967 During

PURIFICATION, ISOLATION AND DETERMINATION OF THE CHEMICAL STRUCTURE

Each venom consists of a great number of components Purification and isolation of the active component(s) must be guided by biological tests

MECHANISM OF ACTION

The natural prey animal may not always be adequate for the determination of the mechanism of action of a venom, because of its size or because too little is known about its anatomy or physiology

In the two species of wasps studies so far, paralysis is caused by an effect on the neuromuscular transmission

A more detailed study of the site of action is in progress but is hampered by the fact that at present the effect of an unknown substance is studied on a neuromuscular transmission process caused by an unknown transmitter substance

T Piek, *Action of the venom of Philanthus triangulum F on the neuromuscular transmission in insects*

Pharmacological Laboratory University of Amsterdam

The venom of the digger wasp *Philanthus* apparently causes paralysis of the skeletal muscles in all insects order and in spiders

Previous investigations by a number of authors as well as by ourselves have shown that it is very likely that the venom causes paralysis by blocking the excitatory neuromuscular transmission

In order to gain more information on the site of action in the neuromuscular junction the effect of the venom has been studied on the spontaneous miniature excitatory postsynaptic potentials (MEPSP s) in the longitudinal flight muscle of the locust *Schistocerca gregaria* Forsk and of the moth *Philosamia cynthia* Hübn These muscles are of the fast type

The venom preparations used are crude extracts of the venom bladders The activity of the different extracts is expressed in Bee Units, based on an estimation of the ED_{50} in honey bees

In *Schistocerca* both the frequency and the amplitude of the MEPSP's are decreased by the venom, but the effect on the frequen

cy is by far the more important In *Philosamia* however, the amplitude of the MEPSP's is not affected, but the frequency of the potentials shows an initial increase followed by a decrease, similar to the effect of *Microbracon* venom in this species (PIEK, 1969)

In both species studied *Philanthus* venom mainly affects the frequency of the MEPSP's rather than the amplitude and therefore may be assumed to act predominantly on a presynaptic site

After administration of *Philanthus* venom, the onset of paralysis of the muscle is speeded up by activity of the neuromuscular system by stimulation of the nerve This may be an indication that the venom affects either the synthesis or the storage mechanism of the neuromuscular transmitter substance

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PIEK T, Acta Physiol Pharmacol Neerl 15 (1969) 87

R T Simon Thomas, *The collection and breeding of wasps producing a paralysing venom*

Pharmacological Laboratory, University of Amsterdam

For an investigation on the mode of action and chemical structure of paralysing wasp venoms large numbers of female wasps must be available, preferably throughout the year Large numbers may either be reared in the laboratory or collected in the field

Parasitic wasps that are suitable for mass culture must fulfil the following requirements 1 mating in a small space, 2 no diapause, 3 large reproduction, 4 easy mass culture of the host

Microbracon hebetor Say (Braconidae), which uses small moth larvae as its host, fulfils all these requirements One female produces an average offspring of about 12 females At present our colony produces 400–500 females daily The following requirements can be formulated for predatory wasps that may be collected in the field 1 easy identification as to species and sex, 2 occurrence in dense populations, 3 good survival during transport

Philanthus triangulum F (Sphecidae) meets these requirements Unfortunately this species has disappeared in Holland, probably following a series of six cool summers from 1961 to 1967 During

PURIFICATION, ISOLATION AND DETERMINATION OF THE CHEMICAL STRUCTURE

Each venom consists of a great number of components Purification and isolation of the active component(s) must be guided by biological tests

MECHANISM OF ACTION

The natural prey animal may not always be adequate for the determination of the mechanism of action of a venom, because of its size or because too little is known about its anatomy or physiology

In the two species of wasps studies so far, paralysis is caused by an effect on the neuromuscular transmission

A more detailed study of the site of action is in progress but is hampered by the fact that at present the effect of an unknown substance is studied on a neuromuscular transmission process caused by an unknown transmitter substance

T PICK, *Action of the venom of Philanthus triangulum F on the neuromuscular transmission in insects*

Pharmacological Laboratory University of Amsterdam

The venom of the digger wasp *Philanthus* apparently causes paralysis of the skeletal muscles in all insects order and in spiders

Previous investigations by a number of authors as well as by ourselves have shown that it is very likely that the venom causes paralysis by blocking the excitatory neuromuscular transmission

In order to gain more information on the site of action in the neuromuscular junction the effect of the venom has been studied on the spontaneous miniature excitatory postsynaptic potentials (MEPSP s) in the longitudinal flight muscle of the locust *Schistocerca gregaria* Forsk and of the moth *Philosamia cynthia* Hubn These muscles are of the fast type

The venom preparations used are crude extracts of the venom bladders The activity of the different extracts is expressed in Bee Units based on an estimation of the ED_{50} in honey bees

In *Schistocerca* both the frequency and the amplitude of the MEPSP s are decreased by the venom, but the effect on the frequen-

In 60 series of different cells in 15 rabbit atria, stimulus frequency was found to be linearly proportional to the logarithm of conduction velocity, while stimulus frequency was also linearly proportional to the maximal rate of rise of the action potential

Changes in sodium inward current might be responsible for con duction velocity changes following an increase or decrease in heart frequency

5×10^{-6} g acetylcholine/ml perfusion fluid increased both conduction velocity and maximal rate of rise At low frequencies acetylcholine had only minor influence on maximal rate of rise and conduction velocity, but at high frequencies both increased considerably

This finding might explain divergent experimental results in literature

B J Visser and W Spanjer, *Biochemical study of two paralyzing insect venoms*

Pharmacological Laboratory, University of Amsterdam

Crude preparations of *Microbracon hebetor* venom, obtained by extraction of isolated venom apparatuses and homogenized whole female wasps contain a large number of components among which amino acids, peptides, enzymes and other proteins No biological activity could be isolated from the starting materials by a) extraction with organic solvents b) sublimation in vacuo, c) ultrafiltration A partial purification could be achieved by a) preparative gel electrophoresis on polyacrylamide, followed by gel filtration on Sephadex G 75, b) gel filtration on Sephadex G 100, followed by ion exchange chromatography on DEAE-Sephadex A 50 The recovery of the biological activity after purification by both methods, however, was still low owing to the low stability of the toxic principle Gel filtration on Sephadex columns calibrated with known proteins, indicated a molecular weight of approximately 55 000

Philanthus triangulum venom preparations, obtained by homogenizing abdomens of female wasps, were partially purified by a series of gel filtrations on Sephadex G 200, G 25 and G 10 in succession Complete recovery of the biological activity after purifi-

1965, 1966 and 1967 an average of 2000 females a year were collected in southwest France

Philanthus can be kept in the laboratory in relatively small cages on top of containers filled with fine sand, if supplied with water, honey and honey bees The females dig breeding-holes and drag paralysed bees into these holes Part of the offspring emerges after 30 days at about 25 °C The remainder can be made to emerge by subjecting them to a 4 weeks' cooling period at 5 °C In the laboratory 1 female produced an average offspring of 1 female and 4 males

The females of the second generation show an apparently normal behaviour However, the offspring consisted exclusively of males, *indicating that no mating had occurred or that possible matings* had been unsuccessful

J W Viersma, L N Bouman and M Mater, *Conduction velocity and rate of rise of the action potential at different heart frequencies in rabbit auricle*

Department of Physiology, University of Amsterdam

The crista terminalis of young rabbits was isolated from the right atrium, without the sino-atrial node The muscle strip was stimulated by means of a bipolar electrode, with programmed, stepwise changing frequencies We used 16 different frequencies between 2 and 9 c p s

The conduction time was measured with two intra cellular microelectrodes, which were placed on the crista terminalis on a straight line with the stimulation electrode The maximal rate of rise of the transmembrane action potential, which can be regarded as a measure for the inward sodium current during depolarization, was calculated

After a sudden change in frequency of stimulation, conduction velocity and maximal rate of rise changed gradually to a steady state value We compared the means of both conduction time and maximal rate of rise for the same cell at different frequencies of stimulation during the steady state

Increase in frequency gives rise to a fall in conduction velocity and to a fall in maximal rate of rise, in close relation The inverse also holds true



This is a special issue of Acta Physiologica
et Pharmacologica Neerlandica

dedicated to Dr Rudolf Magnus,

commemorating the 60th anniversary of the Chair of Pharmacology
of the Medical Faculty in the University of Utrecht

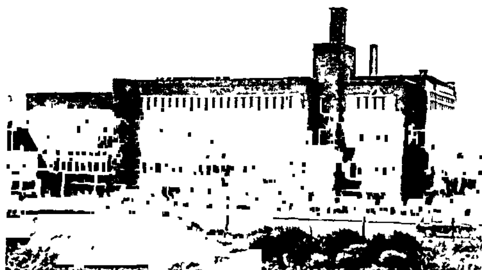
cation was achieved *Philanthus* toxin appeared to be a more stable molecule than *Microbracon* toxin presumably having a molecular weight of less than 700 (provided no retardation takes place because of adsorption during the gel filtration)



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The former Department of Pharmacology, now called "Rudolf Magnus Institute for Pharmacology" The laboratory was built with the aid of the Dutch Government and the Rockefeller Institute in 1925 and opened in 1928

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PREFACE TO THE RUDOLF MAGNUS ISSUE

On November 8th, 1968, the 60th anniversary of the creation of the Chair of Pharmacology of the Medical Faculty of the University of Utrecht, the first in the Netherlands, was celebrated In 1908, Doctor Rudolf Magnus from Heidelberg who was then 35 years old was appointed as the first professor of pharmacology Upon his arrival Magnus was already well known for his work but he became famous during his stay in Utrecht His work on the postural reflexes, in collaboration with the otologist De Kleijn, is still a landmark in physiology

The University of Heidelberg asked him to return to his Alma Mater when he was at the height of his fame The fact that the Rockefeller Foundation and the State of the Netherlands were willing to supply the money to build a laboratory exclusively for pharmacology helped Magnus to decide to stay in Utrecht Unfortunately, he died in 1927 just before the opening of his laboratory which was at that time one of the most modern in the world One of his former co workers, Dr U G Bijlsma, succeeded Magnus in 1929 and worked in the laboratory for 35 years Many pharmacologists received their training at this time and during Professor Bijlsma's office, pharmacology gradually developed into a multidisciplinary science This made it desirable to rebuild the laboratory and to re-equip it although this was done after his retirement as head of the department Upon completion, the Board of the University of Utrecht granted permission to name the laboratory after Rudolf Magnus in honour of a great scientist.

On November 8th, 1968 Magnus' grandson, Jan Rudolf Magnus, unveiled the stone bearing the new name "Rudolf Magnus Institute", in the presence of a number of government and university officials members of the Magnus family and many colleagues

On November 9th, 1968, a symposium was held under the auspices of the Netherlands Society for Physiology and Pharmacology to mark the 60th anniversary This symposium was intended to be a survey of a number of topics of research currently under investigation in the various laboratories of Physiology and of Pharmacology in the Netherlands The papers given at the sympo-

sium, are published in this issue of Acta Physiologica et Pharmacologica Neerlandica, together with the two lectures on Rudolf Magnus and on the history of the Chair of Pharmacology by Rudolf Magnus' son, Dr Otto Magnus, and by Professor Bijlsma

I gratefully acknowledge the courtesy of North Holland Publishing Company for giving us the opportunity of editing this Rudolf Magnus issue and I also wish to thank all the authors for their very valuable contributions

Utrecht, November 1968

D DE WIED M D
Professor of Pharmacology
Chairman of the
Rudolf Magnus Institute

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MAGNUS AS SCIENTIST AND TEACHER

BY

U G BIJLSMA

The part which I have to play in this meeting dedicated to the commemoration of the 60th anniversary of the creation of the chair in pharmacology, is that of the biographer of the first twenty years of this period, when Rudolf Magnus was the first full professor in pharmacology

Classical pharmacology has been concerned with the task of answering the question of why a certain drug exerts its known therapeutic action Until the middle of the last century this was the domain of the clinician who tried to find the answer from his observations in patients, and who also tried to formulate the correct indications

On previous occasions when I have spoken of classical pharmacology, the term has been interpreted by some people as meaning old fashioned or even obsolete pharmacology This is not fair Even if a substance is suspected of being a useful drug on the basis of animal experiments, it is still clinical pharmacology which provides the final practical and theoretical proof

In the second half of the last century, scientists started to consider these fundamental questions They discovered that experimental research in animals, in which a greater variety of experiments are possible, provided a better insight into the actual problems

The prominent names of Claude Bernard and of Buchheim should be mentioned here, with their difference in starting points Bernard the physiologist and Buchheim the clinician, each with a different attitude, nevertheless approached each other in their work Since then experimental pharmacology has taken shape and constitutes a part of classical pharmacology just as clinical pharmacology does

Utrecht soon acknowledged this situation When in 1868 Engelman was appointed as the fifth professor in the medical faculty, one of his many tasks was experimental pharmacology Thus,

we now also commemorate the fact that the word pharmacology has been present in our curriculum for exactly one century.

One year later followed the appointment of a lecturer, Brondgeest for "experimental pharmacology, physical and propadeutic diagnosis and the theory of animal heat production in the healthy and the ill state", the last part may be considered as the start of pathological physiology. Brondgeest obtained a two roomed laboratory in 1896, and died in 1904. Then experimental pharmacology vanished silently, and two lecturers were appointed for pharmacognosy and clinical pharmacology.

In 1908 Magnus was appointed as professor in pharmacology, without any other subsidiary tasks. A suitable laboratory was soon found in the house "Leeuwenbergh", which had been founded by Agnes van Leeuwenbergh as a pesthouse, but had never been used as such. It had served as quarters for the body guards of King Louis Napoleon, and later on as a chemical laboratory. The building was somewhat changed and improved, but the animal housing was very poor, the roof was leaking and the rafters had to be supported. From 1920 Magnus refused each year to start his lectures, unless a declaration was given by an expert that attendance of his lectures had not yet become dangerous. By that time Magnus had a number of scientific and technical assistants and even a secretary with a typewriter.

In his teaching he followed the method of Schmiedeberg. He abandoned the botanical aspects of pharmacognosy and concentrated on the most important aspect of drugs their mechanism of action. For pre clinical students this was restricted to the normal healthy organism and for clinical students he combined the teaching of pharmacology with pathophysiology.

His scientific program was a logical consequence of his work in Heidelberg where he had been working with Gottlieb. However, he had been working in other laboratories temporarily e g the Zoological Station in Naples and he had also visited several laboratories in England and other countries. These visits had made him many friends among whom Sherrington and Schafer, particularly the former had directed his attention to the nervous system.

The work of Magnus before 1908 included several important investigations e g experiments on salt and water metabolism the pharmacology of heart and circulation, and the gastro intestinal

tract In Utrecht many studies were performed on purgatives made possible by the application of X rays From there the synergistic and antagonistic actions of several opium alkaloids were studied and questions of tolerance and addiction were analyzed Heart and circulation were investigated with the aid of new techniques heart irregularities and coronary diseases received attention and also pathological physiology was further developed

An important theoretical question was whether spontaneous rhythmic movements were completely neurogenic in origin In Utrecht the fight between a neurogenic and a myogenic theory of the heart rhythm was not yet settled and Magnus decided to use the intestines as a model since it appeared to be impossible to remove the innervation from the heart Together with Boeke he attacked this basal question until it also appeared impossible to separate muscular and nervous elements in this tissue In the meantime it had been found that the gut produced a substance which stimulated intestinal movements and this substance appeared to be choline a finding which was one of the new facts which led Loewi to formulate the humoral transmission of neural stimuli

Even before 1914 the induction of pulmonary edema by mechanical and toxic influences was studied and during the first World War Magnus and Laqueur studied the effect of the war gas phosgene and the therapy of its intoxication during the time when as Germans they were mobilized and had to serve in the army

During his absence his staff members especially Storm van Leeuwen made an intensive study of the quantitative aspects of drug antagonism Later on Magnus sought the assistance of the professor of astronomy for the mathematical approach to these problems

Although his contributions to pharmacology were important he received public recognition chiefly for his work on the physiological meaning of postural reflexes The integration of motor reflexes labyrinth physiology and brain centers involved in motor behavior were analyzed by Magnus and his co worker De Kleyn in their experimental work on locomotor and postural movements

Not only in his scientific work but also in the design of the present building Magnus displayed a great talent for systematic work and an ingenious vision of the development of research His

untimely death prevented him from actually working in this laboratory

I come now to the second and more difficult task an evaluation of Magnus as a person The facts related up to now can be checked by anybody, merely by studying the documents Very few living people, however, knew him personally and worked with him Of course, a personal recollection of the man Magnus is subjective, but *I will try to give a fair impression of him I think we could* approach him best by asking ourselves how he achieved such eminence

It is obvious that he was a man of great intellect, but intellect can have many forms, and a more discerning estimation has to be made

Magnus read much and fast, pharmacology, physiology, pathophysiology and philosophy, and he was capable of separating rapidly the important from the insignificant Moreover, he had an excellent memory, and he could incorporate every new fact into the framework of the concepts he already had in his mind His habit of making notes of his reading and inserting these in his lecture material was very useful In this way not only were his lectures up to date, but also he was able to correct a doubtful theory One of his aphorisms was a scientific truth lives only for fifteen years

His lectures were critical, detailed and documented, which meant that they were difficult for the students He did not care that attendance at his lectures was rather low, because he gave them for the students who were interested in scientific reasoning and research

His phenomenal memory was a help to his co-workers for at the time only a few old abstracting journals were available Since the *library was in his office we only had to venture a modest sigh* and he would ask what is it you are looking for? Take that journal, about that volume, and you will find your information

His attitude towards his pupils was that of a father towards his inquisitive sons He acted according to a proverb of Confucius he who works as a scientist and does not love people is like some body who lights a torch and closes his eyes

The results of all experiments were discussed, and then came the question what are you going to do next? His advice was given

in such a way that the pupil was convinced that he himself had found the answer, and often this was true because Magnus had forced him into logical reasoning

He behaved as a father of studious sons and of course, as amongst real sons sometimes the different characters and convictions caused conflicts How wise he was in such cases! None of us could tell from his attitude or words whether he had any preferences, he never told anyone that he disapproved of something done by someone else Approval or disapproval were expressed only in private In this way a good relationship was maintained between his staff and also his guests from other institutes

I have been asked sometimes whether Magnus was ambitious Apparently his ambition did not show clearly Was it hidden, or is hidden ambition no ambition? On the other hand is ambition not necessary in order to perform a task so well? Without any ambition there is no need to publish the results of research. Perhaps one should not speak of ambition, but of drive or motivation

At any rate, his pupils respected him very much, and Magnus had many friends amongst the pharmacologists, physiologists and neurologists His modesty must have prevented him from being proud of the homage paid to him by many distinguished scientists

Shortly before his death he was considered as a candidate for the Nobel Prize, and to judge from the comments given by the examiner, only his death prevented him from receiving the award It seems appropriate to conclude this commemoration with a literal citation from the book "Nobel the man and his prizes", which summarizes the judgment made in 1927

"The investigations of R Magnus and A de Kleyn referred to above concerned tone and posture in different circumstances It was found that the rigidity developing in decerebrate animals after trans section of the brainstem especially in their limbs, depends to a great extent on the position of the head A more detailed analysis revealed that it was a question of tonic reflexes which are affected partly by the position of the head in space and partly by its *position in relation to the* neck Both groups, which can reinforce or weaken each other according to a definite pattern have been combined under the name of attitudinal or standing reflexes since they enable the animal to stand up These reflexes can also be observed in normal, intact animals, in all habitual

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RUDOLF MAGNUS

PERSONAL REMINISCENCES

BY

O MAGNUS

On this 60th anniversary of the creation of the Chair of Pharmacology in Utrecht, it is my privilege to try to give an impression of the personality of my father Rudolf Magnus, who held this Chair from 1908 until his death in 1927

He always liked to use quotations and aphorisms One of them was a quotation from Goethe's Faust "Was du ererbt von Deinen Vätern hast, erwirb es um es zu besitzen!" (What you have inherited from your ancestors you must acquire in order to possess it)

Indeed when he was born on September 2, 1873 in Brunswick, Germany he was gifted with qualities which he must have inherited from both his parents His father was a lawyer and notary in Brunswick, his grandfather and great-grandfather were doctors His literary talents he probably *inherited* mainly from his mother Her father was librarian of the municipal library in Hamburg

After he had left the gymnasium in Brunswick he intended to study literature But a friend of his father, Richard Meyer, the brother of the famous Victor Meyer, then professor of chemistry in Heidelberg, convinced him that he should not waste his intelligence on the past, but should rather choose a scientific career Accordingly he went to Heidelberg to study medicine There he was particularly impressed by the lectures of the physiologist Kühne and we still have in our possession a neatly bound volume with the notes he made during Erb's lectures in neurology He completed his medical studies in 1896 and obtained his Ph D in medicine summa cum laude in 1898

In those days a scientific career was even less lucrative than at present He therefore first asked his father whether he was willing to continue to support him as far as was necessary Fortunately this support was gladly provided and he became assistant to the

movements they play an important part. While a decerebrate animal can stand up but is not able to get up on its feet, it can get up if the cerebrum alone has been removed, provided the big nerve centers or ganglia situated at the base of the brain are left intact. This ability is due, as Magnus and De Kleijn discovered, to a special group of "righting reflexes" which are elicited, partly by the vestibular apparatus *in the inner ear and the neck*, partly by the eyes, and, partly, by the trunk of the body. It is these complex reflexes which enable a cat always to land on its feet. Obviously, they are also of the utmost importance in man."

The works of Magnus and De Kleijn were declared by the examiner (1927) clearly to deserve a prize, and the prospects for an award seemed most favourable when Magnus died unexpectedly.

In my inaugural address in 1928 I said that the name of Magnus will always be connected with this Institute. It is a pleasure and an honour to act now as the advocate of the new name of this laboratory.

scientific publications and notes enabled my father to study these very extensive investigations in greater detail This led him to ask permission to repeat Goethe's experiments with the instruments Goethe had used in his house in Weimar He was able to confirm that Goethe s observations were completely correct In 1906 he gave a series of 10 lectures on "Goethe als Naturforscher" ("Goethe as a scientist"), these lectures were published as a book in the same year

The first lecture began with a poem of Goethe which I should like to quote, because it was very characteristic of my father's approach to science

"Weite Welt und breites Leben
Langer Jahre redlich Streben
Stets geforscht und stets gegrundet
Nie geschlossen oft gerundet
Aeltestes bewahrt mit Treue
Freundlich aufgefasstes Neue
Heitern Sinn und reine Zwecke
Nun! man kommt schon eine Strecke"
(Wide world and broad life
Long years of honest strife
Always searched and always tested
Never completed often improved
Old experience guarded faithfully
A friendly reception to new things
A cheerful mind and a clean purpose
Well! one may hope to make some progress)

Relations with Professor Gottlieb were apparently not always good which may have been due to the fact that the pupil began to receive more recognition than the master Therefore my father accepted with pleasure the invitation to become the first professor of pharmacology in Utrecht He received the announcement of his appointment in a hand written letter from the secretary of the Board of Governors of the University We do not know what he thought about his salary of ƒ 4 000 (approx $ 1 100) a year and of the two 5 yearly increases of ƒ 1 000 ($ 275), but we do know that my father never regretted having come to Utrecht He and my mother soon felt at home there, they had a large circle of

professor of pharmacology Gottlieb in Heidelberg But from there he traveled abroad and spent several periods in various laboratories abroad, i a in the zoological station in Naples, with Schafer in Edinburgh and, most important of all for his future work, with Sherrington in Liverpool

In Naples he met the biologist Von Uexkull who soon became his friend He was impressed by Von Uexküll's observation in invertebrate animals that the distribution of the excitability of nerve centres depends on the state of contraction of the muscles they innervate In Sherrington's laboratory he wanted to investigate whether similar reactions could be demonstrated in vertebrates He therefore made observations on the tail of a dog after a spinal transection This did not prove a very satisfactory preparation, but during these experiments his attention was drawn towards the postural reactions in the hind legs of the dog From then on it was decided that he should make a special study of the postural reflexes which Sherrington then left to him to elucidate However, they kept in regular contact

In 1896 my father met my mother who came from a family with many interests in arts Her home was a centre for artists in Munich It was characteristic both of my father and of the time in which he lived that he waited to ask my mother to marry him until he considered himself to be in a position to sustain a family Fortunately my mother was prepared to wait six years for his proposal Their marriage was a very happy one Here it should certainly be mentioned how much my father owed to his wife She always created the best circumstances for his work and his other activities She not only took care of the education of the children and the ordinary household duties but also of all business matters Moreover she was always willing and prepared to receive the many visitors – even if they were announced just before lunch or supper.

In 1901 my father was given the "venia docendi" in Heidelberg, he became an "extra ordinary" (associate) Professor in 1904, at the age of 31

Besides his ordinary professional work he did not lose his literary interests He had a great admiration for Goethe, not only because of his literary work, but particularly because of the universality of his genius A publication of the complete work of Goethe in Weimar, which for the first time made available the complete

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friends in Utrecht as well as in other parts of Holland and abroad.

During the first world-war he was called back to Germany to do military service. But on repeated requests from the Dutch government he was released in 1917 in order to take up once more his position as professor of pharmacology, which during his absence had been filled very ably by the deputy director of the Institute W. Storm van Leeuwen Sr.

One of my earliest memories is of the disappointment I felt when I was not allowed to accompany the other members of the family to the railway station from where my father was taken home by the Utrecht students in a torch procession.

After the war he resumed contacts with his English and American friends and colleagues such as Sherrington, Dale and Cannon.

In 1924 his monograph "Körperstellung" ("Posture") appeared. One year later he was invited to return as a professor to Heidelberg. There he was offered a modern laboratory instead of his beloved old laboratory Leeuwenbergh, which had become much too small. When however, the Rockefeller Foundation offered to contribute half of the costs of a new laboratory in Utrecht, provided the Dutch Government would contribute the other half and this offer was accepted, my father decided with pleasure to stay in Utrecht amidst his team of collaborators and his many friends. Apparently he and his family were not the only ones who were pleased; when the decision to remain in Utrecht became known, the students celebrated this with a serenade, during which their president P. Lieftinck (who was later to become a prominent finance minister and financial expert of international fame) interpreted the students' pleasure.

We still remember the ceremony of the laying of the first stone of the new laboratory by his youngest daughter Erica (now Mrs. Burdon). However, my father did not live to attend the opening. During a holiday in his beloved Pontresina in Switzerland where he was making preparations for a journey around the world, including visits to the United States and Japan, he suddenly died in the night between the 24th and 25th of July 1927. It would certainly have filled him with pride and satisfaction if he could have known that this Institute would be named after him.

Let me try finally to describe his personality and to analyze the qualities that enabled him to create a body of scientific work

which apparently is not outdated after 40 years It seems to be an exception to the rule of Von Uexkull which he often quoted "Was ist eine wissenschaftliche Wahrheit? Ein Irrtum von heute" (What is a scientific truth? An error of today)

Professor Bijlsma has already mentioned his intelligence and excellent memory I should like to add that both were highly selective Matters which he thought of no importance or which did not interest him he immediately forgot He had apparently a gift for what he himself characterised as the essence of scientific research "Man muss sich am richtigen Augenblick wundern" (One must be surprised at the right moment) His literary talents were of great use to him, both in writing and speaking he could formulate his thoughts clearly and easily and used to dictate a lecture or an article directly in its final form Already in 1920 he used a dictaphone for this purpose The chapters of the "Korperstellung" were dictated in the same way

He was extremely well organised, could concentrate completely on what he was doing and did not allow himself to be distracted Moreover, he was very punctual both for lunch and supper he came home exactly on time and expected his children not to be late During meals each of the four children reported in turn what he or she had experienced and at these times his attention was focussed entirely on the family On winter evenings he often read to the family after supper for half an hour from works of his favorite writers Afterwards he worked in his study until 11 o'clock and could not be disturbed However, frequently he had to attend meetings and quite often my parents received guests in the evening He enjoyed social life, good food and a glass of wine On these occasions his sense of humour and his gift as raconteur and story teller contributed much to the congenial atmosphere

There was not much time for hobbies Apart from his work he liked walking, particularly in the mountains, and in winter he was a keen skater Sometimes when there was good ice on the canals of Holland the whole laboratory had a day off for skating I do not know whether this tradition is still kept alive, perhaps it can be reintroduced with the new name of the Institute

Results of scientific research are often considered to be a purely intellectual achievement Intelligence is necessary, but not sufficient Qualities of character and personality and certain social qualities

are as essential as intelligence Reliability, a sense of responsibility, perseverence, are only a few of them Apart from these my father had a particularly good and natural contact with nearly everybody whom he met This was mainly based on the fact that he had a notably well integrated personality This was evident in the great number of his friends and in the devotion of his collaborators and pupils It is still evident today when we meet somebody who knew my father Nearly always some touching memories come to the fore My father himself, together of course with my mother profited most from these good personal relations

When reading once more the many eulogies which appeared after his death it struck me that some of his best friends wrote that he was a happy man As such we also remember him

Before finishing I should like once more to quote one of his aphorisms "One should make a work of art out of one's life" I think we are justified in stating that he accomplished this mission

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THE REGULATION OF ACTH SECRETION

BY

P G SMELIK

This paper is not intended to be a general survey of the literature on the regulation of ACTH secretion but rather a review of our own work For a more extensive review of recent literature the reader is referred to the excellent chapters written by FORTIER (1966), MANGILI *et al* (1966), and YATES (1967)

Soon after it first became clear that the secretion of adrenal cortical hormones was controlled by corticotrophin (ACTH) from the anterior pituitary (SMITH, 1930), the question was posed (as formulated by WALTER B CANNON in 1937) "If the pituitary regulates the adrenal cortex, what regulates the pituitary?" (INGLE, 1959)

A simple model for such a control, devised by SAYERS and SAYERS (1947), was that the level of circulating adrenocortical hormones would influence directly the rate of ACTH secretion The basic idea was, that during stress more corticoids will apparently be needed, the increased tissue utilization will decrease corticoid blood levels, and this decrease will in turn stimulate the ACTH producing cells to secrete the hormone Conversely, an increase *in* the corticoid levels will inhibit ACTH secretion, so that a *true* negative feed back system would be operating

There are strong arguments in favor of the existence of such a feed back system Adrenalectomized animals develop an extremely high titer of circulating ACTH, conversely, administration of corticoids inhibits ACTH secretion

However, for several reasons this theory very soon appeared to be incorrect in the case of acute stressful stimulation In the first place, when it became possible to measure corticosteroid levels in plasma directly, no decrease at all was noted in corticoid con

centrations after the application of a stress stimulus. Secondly, the release of ACTH occurs within minutes after an acute stimulation, much faster than could be accounted for by the corticoid feed-back system. Moreover, it soon became clear that in animals and particularly in man, emotions like fear, fright and anger are much more effective in provoking a fast reflex ACTH secretion than bodily damage or muscular effort. The entrance of the physician, the sight of an accident, the impact of an explosion all result in an immediate release of ACTH, and in such cases it is not at all clear why an increased need for, or utilization of adrenocortical hormones should exist. When adrenal ascorbic acid determinations were used as an index for adrenal activity a group of rats might be transferred from the animal quarters to the experimental room and wait there for some time in order to undergo some stressful procedure. However, soon after plasma corticosterone determinations became possible, it appeared that even opening the door of the rat cage, and certainly the transfer of a rat to another environment, constitutes an acute stimulus for ACTH secretion – a fact which has embarrassed many experimental workers on stress, and still had to be recognized by some workers even in recent years (FRIEDMAN and ADER, 1967). We have been using transfer to a strange environment since 1959 as a stressful procedure, and the pituitary-adrenal response to this emotional stimulus appears to be dependent on the integrity of the hypothalamus (SMELIK, 1959) and the posterior lobe of the pituitary (SMELIK, 1960; DE WIED, 1961).

Following the work of HARRIS (1948), HUME and WITTENSTEIN (1950) and McCANN (1953) it became apparent that the hypothalamus is intrinsically involved in the control of pituitary-adrenal function. This discovery initiated the development of a new field, that of neuroendocrinology, in which the analysis of the nervous control of the endocrine system constitutes the central problem. This paper will deal only with work pertaining to this subject, and will not review our studies on the role of the posterior lobe or on the distinction between "psychic" and "somatic" stress (for review see SMELIK *et al.*, 1962; FORTIER, 1966).

Concerning the hypothalamic control of pituitary-adrenal function, it has now been established that, since any significant neural connection between hypothalamus and anterior pituitary is absent,

humoral factors, produced by the hypothalamus and transported via the portal vessel system are responsible for the regulation of pituitary function The idea that neural tissue is capable of elaborating blood borne factors ("neurosecretion"), is widely accepted, perhaps somewhat too readily by endocrinologists, who were tempted to consider the hypothalamus as another endocrine master gland I would like to recall here that the Nobel laureate W R Hess, in a dinner speech as the Honorary President at the Symposium on the Diencephalon in Milan, 1956, showed some embarassment after hearing so many papers on *hypothalamic factors*, and stated emphatically that, just as a woman is more than mammary glands, the hypothalamus is more than an endocrine gland Looking back to those days, it is no longer so amazing that of all noxious stimuli, emotional disturbances most readily activate the pituitary adrenal system The hypothalamus can be considered as a nodal point between the limbic rhinencephalic and mesencephalic circuitry, which is involved primarily in affective and emotional behavior (PAPEZ, 1937, NAUTA, 1960) Incoming stimuli from enteroceptive as well as proprioceptive receptors will be conveyed to the hypothalamus, which is the integrating and coordinating center for the vegetative or autonomic reactions in response to every disturbance of the homeostasis of the organism I will return to this point later, but conclude now that neuroendocrinological research has the task of unravelling the integrative function of the brain stem in respect to the autonomic system and the endocrine system

In view of the fact that the existence of a specific hypothalamic factor controlling ACTH secretion (Corticotrophin Releasing Factor, or CRF) cannot be denied, the question of the control of the pituitary adrenal axis has shifted to the hypothalamus The two main problems here have been 1 what is the identity of the CRF? 2 what is the site of action of the corticoid feed back?

It should be clear that these questions are closely related In fact it seems impossible to solve either of them completely when the other is still unsettled Since by *definition* the CRF will act *directly on the anterior* pituitary even when the hypothalamus has been eliminated, corticoids should be able to block its action if they act directly on the pituitary, but the effect of CRF should not be prevented if they act on the hypothalamus Consequently, blockade of the effect of a CRF preparation by corticoid administra-

tion can be taken as evidence either for a pituitary site of action of the corticoids or as evidence that the CRF preparation under study does not contain the true physiological CRF A considerable amount of controversy has arisen in the literature because one investigator tried to prove that this CRF preparation contains *the* CRF by injecting it into a corticoid blocked animal whereas others have tried to detect the site of action of corticoids by injecting CRF preparations

SITE OF ACTION OF CORTICOSTEROIDS

Some years ago it was thought that the question of a hypothalamic or pituitary site of corticoid feed back action could be solved by implanting a small amount of a corticoid into these structures themselves and then measuring pituitary adrenal responses to stress Implantation work in the rabbit revealed that inhibition of adrenocortical reactivity could only be obtained if the steroid was implanted into the hypothalamic region between the optic and the anterior part of the median eminence but no inhibition was seen if the implants were put in the pituitary or elsewhere in the brain stem (SMELIK and SAWYER 1962) This was taken as evidence for a hypothalamic action of corticoid feed back Corroborating results were obtained in the rat (DAVIDSON and FELDMAN 1963 CHOWERS *et al* 1963 CORBIN *et al* 1965 SMELIK 1965) All authors now agree that the most effective site for implantation is the anterior part of the hypothalamus

However serious objections can be raised against the interpretation that the site of action must be the hypothalamus Interestingly enough DE WIED (1964) demonstrated that the effect of CRF preparations could be blocked progressively by pretreating the animals with increasing doses of dexamethasone a synthetic corticosteroid Even in animals in which the hypothalamus had been destroyed (and in which the CRF preparations were effective indicating their action on the anterior pituitary) dexamethasone was capable of blocking their effect This strongly suggests that corticoids if administered in sufficient amounts can act directly on the pituitary

Moreover from a theoretical point of view it has been argued by BOGDANOVE (1963) that perhaps the anterior basal hypo

thalamus is the most efficient site for implantation because the hormone is deposited close enough to the portal vessel system to be transported to the anterior pituitary, in this way the ACTH producing cells would be more easily reached by the steroid than by implantation of the crystalline substance in the anterior lobe tissue itself Bogdanove's idea of an "implantation paradox" appeared very difficult to prove or disprove Personally, our impression from the data now available is that the idea is ingenious but not correct Implantation will cause a local saturation with the hormone, and if the only site of action were the anterior lobe it is difficult to understand why pituitary implants have no effect at all One would expect a gradient directly related to the degree of contact with the primary plexus of the portal vessel system Our studies with corticosterone implants in rats did not verify such an expectation The most effective site of implantation was not the one closest to the portal vessel system but the region between optic chiasm and median eminence The inhibitory action of implants decreased proportionally to increasing distance from this region (Fig 1)

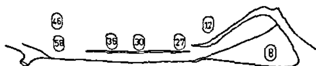


Fig 1
°₀ Inhibition of stress response by corticosterone implants

Theoretically the most decisive evidence would be obtained from an experiment in which a CRF preparation is administered to an animal bearing a hypothalamic corticosteroid implant If the effect of CRF were blocked by such an implant, it could be concluded that the block is at the pituitary level We have attempted such an experiment with dexamethasone implants and the subsequent administration of a crude, but vasopressin free CRF preparation which appeared to be active in many assay preparations for CRF activity (see below) It appeared that the effect of this crude CRF was not blocked (although somewhat diminished) by hypothalamic dexamethasone implants whereas the effect of posterior lobe principles was completely abolished (Fig 2)

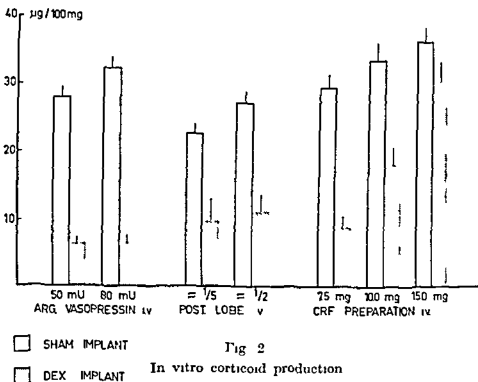


Fig 2
In vitro corticoid production

In view of these and other results we believe that specific corticosteroid elements are situated in the anterior part of the hypothalamus the same area as is thought to control pituitary adrenal function by elaborating the CRF

This does not imply however that corticoids cannot block pituitary tissue at all The question here is whether this happens when corticoid levels are elevated within the physiological range The available evidence strongly suggests that this is not the case The most direct evidence for a pituitary site of action has come from the observation that at least 100 µg of dexamethasone has to be administered subcutaneously in order to prevent CRF induced ACTH release and the effect of this dose of dexamethasone clearly cannot be matched by any increase in concentration of endogenous corticosteroids (DE WIED 1964)

Recently it has been found that microinjection of 4 µg of dexamethasone phosphate directly into the anterior pituitary prevents stress induced ACTH secretion (YATES personal communication) In earlier studies (with a more sensitive strain of rats however) ACTH release due to a scald stress could be blocked by

2 μg intravenously or 10 ng injected into the median eminence or anterior hypothalamic septal region (DALLMAN and YATES 1968) These results also suggest that the pituitary is sensitive to corticoids but that the most sensitive area is located within the central nervous system

In conclusion it is thought that the primary site of action of corticosteroid feed back is the anterior hypothalamus, and that only pharmacological doses of corticoids are capable of blocking pituitary tissue as well This would imply that the steroid blocked animal (if the corticoid is administered systematically) is not a suitable preparation for CRF assays The degree of pituitary inhibition will of course depend on dose route of administration and time but it is questionable whether any procedure will escape the Scylla of incomplete protection against unspecific stimulation and the Charybdis of decreased pituitary responsiveness

PHYSIOLOGICAL ROLE OF CORTICOSTEROID FEED-BACK

Some years ago an ingenious attempt was made to revive the idea that the level of blood corticoids continuously controls the activity of the pituitary adrenal system The theory advanced by YATES and associates (1961) requires that a 'comparator" exists situated in the central nervous system which is capable of detecting a discrepancy between the actual corticoid levels and the demand for corticoids in that situation and which consequently will reset the setpoint of the controller The attractiveness of the theory lies in the fact that such a system would provide a continuous automatic control which can operate in every situation

Unfortunately the theory is not entirely acceptable We have shown that in case of acute stress the reflex activation of the pituitary adrenal axis is independent of the circulating corticoid levels unless they reach supra physiological values (SMELIK, 1963a) Accordingly the results of YATES *et al* (1961) can be explained by a failure to recognize these supra physiological levels in their experiments

Study of the time-dosage relationships appeared to disclose another important aspect of the feed back mechanism When a supra physiological level of corticoids is provoked by intravenous

injection of corticosterone inhibition of stress induced ACTH secretion occurs within 15 sec and lasts for about 20 min However if corticosterone is given subcutaneously the inhibition develops only after two hours and lasts for about 8 h during which inhibition plasma corticosterone levels have already decreased to normal values (SMELIK 1963b) Furthermore if a comparable rise in blood corticoid titers is induced by intraperitoneal injection of corticosterone the blockade *appears within 30 min and is present* for 60–90 min If similar experiments are performed with dexamethasone instead of corticosterone the inhibitory action appears later and lasts for a longer period of time

These results indicate that a time delay exists between peak corticoid levels and onset of inhibition and this delay is mainly dependent of the route of administration A slower resorption of the corticoid is also followed by a longer duration of the blockade Thus not only the absolute concentration in the blood but also the rate of appearance in the blood seems to be important This suggests that a corticoid sensitive site slowly accumulates the steroid and that inhibition is proportional to the degree of local binding at the feed back site A preliminary working hypothesis has therefore been put forward that the inhibition is a function of the product of time and concentration (SMELIK 1963a b) This conclusion has also been accepted by YATES (1967)

In summary corticoid feed back would appear to dampen the hypothalamo pituitary adrenocortical system but to possess a rather limited capacity If no corticoids are present (as in adrenal ectomized animals) the system idles at a high level the basal production of ACTH is elevated and activation of the system by stress provokes an excessive reflex ACTH secretion The presence of a functional adrenal cortex keeps the system in check under normal conditions the low concentration of corticoids in the blood require a long time to exert this inhibition and the capacity will only be sufficient to control slow and small variations in the external milieu reflected by diurnal shifts in activity Whether reflex ACTH secretion will occur will depend on the intensity of the disturbances and the actual corticoid levels However these levels can never be increased by the adrenal cortex to a level sufficiently high to block the system completely Administration of pharmacological doses of corticoids can accomplish this but the blockade

is then extended to the anterior pituitary itself, rendering the pituitary unresponsive to CRF Stressful stimuli will activate the pituitary adrenal axis seemingly irrespective of the existing corticoid levels, only extreme situations like adrenalectomy or treatment with pharmacological doses of corticoids will disclose that they do influence the rate of ACTH secretion

Future research should concentrate on the role of the endocrines in the adaptation to the demands of everyday life, rather than on models in which such extreme conditions are provoked Further refinements in methodology and determinations will certainly disclose continuous automatic adjustments of autonomic, metabolic and behavioral regulations by the target hormones

MECHANISM OF ACTION OF CORTICOID FEED-BACK

Very little is known about the actual mechanism through which corticoids modulate the center responsible for the control of ACTH secretion Since they seem to act primarily on the anterior part of the hypothalamus, a site which is thought to elaborate the CRF, the most obvious hypothesis is that they prevent in some way the production and/or release of CRF If one assumes that CRF is produced by special neurons, which have their cell bodies in the anterior hypothalamus and send their axons to the median eminence corticoids might act by rendering these cells insensitive to afferent neural stimuli, by inhibiting the electrical activity of these cells by interfering with the biosynthesis of CRF, by preventing the transport along the axons or by blocking the release of CRF from the nerve endings

An inhibition of CRF-synthesis has been suggested by VERNIKOS-DANELLIS (1965) RUF and STEINER (1967) have found that dexamethasone inhibits the rate of firing of some hypothalamic cells

Recently we have observed that after establishing a blockade by dexamethasone either direct mechanical stimulation of the anterior hypothalamus or administration of vasopressin is still capable of inducing a small and short lasting adrenocortical activation This would suggest that after corticoid treatment some CRF is still present which can be released by direct stimulation Accordingly, we have assumed that dexamethasone blocks

the production rather than the release of CRF, and that under these conditions a neuronal pool containing releasable CRF can still exist Further experiments appear to support this idea If the pool is depleted by appropriate stimuli during dexamethasone inhibition vasopressin or mechanical stimulation are no longer capable of causing a transient ACTH release A CRF preparation is still active under these circumstances (HEDGE and SMELIK, 1969)

These results suggest that dexamethasone interferes with the elaboration of new CRF, but do not indicate how this is accomplished At the same time they suggest that vasopressin does not act as a non specific stress stimulus or as a CRF, but rather has a direct effect on the release of CRF from the pool within the CRF neurons

NEURAL INPUTS TO THE HYPOTHALAMIC CONTROLLER

Up to this point it has been taken for granted that stressful stimuli in some way reach the CRF producing neurons It is conceivable that different stimuli converge in the hypothalamic area where the cell bodies of these neurons are situated, since the hypothalamus receives afferents from many structures (medial forebrain bundle, fornix stria terminalis bundle of Schutz, etc) Nevertheless the nature of such synaptic contacts is not known although some evidence exists for cholinergic and for noradrenergic transmission in this area (SHUTE and LEWIS 1966, ZAMBRANO, 1968)

We have studied the effect of reserpine implants in the hypothalamus in order to obtain some information about the role which monoamines might play in the transmission of neural signals to the regulating center Local depletion of monoamines in this region by reserpine did not modify the ACTH secretion in any respect studied It was therefore concluded that no evidence could be produced indicating an obligatory role of monoamines in the control of ACTH release (SMELIK 1967), although they do participate in the control of prolactin secretion (VAN MAANEN and SMELIK, 1968)

In studying the effect of several autonomic drugs it was found that hypothalamic implants of atropine prevented stress

induced ACTH secretion The effective site for implantation appeared to be restricted to the anterior part of the hypothalamus, inhibition occurred within 30 min after implantation (HEDGE and SMELIK 1968)

This suggests that a cholinergic link is involved in the activation of hypothalamic centers controlling ACTH secretion In view of the localization it is conceivable that the CRF neurons are activated via a cholinergic synapse There are some indications in the literature that a similar situation may exist for the neurosecretory cells producing vasopressin (ABRAHAMS and PICKFORD, 1956, WALKER, 1956) This would be consistent with the apparent close relationship between the control of ACTH and of ADH secretion

THE STRESS RESPONSE

A few speculative words may be said about the over all system which mediates the response to acute noxious stimulation The neuro endocrine reaction to imminent danger and bodily damage seems to subserve a dual purpose first, a mobilization of energy sources and activation of the systems involved in muscular effort, second repair of the damage inflicted and replenishment of depleted energy sources

The autonomic nervous system is the mediator for these reactions The immediate reflex response consists of excitation of the sympathetic system resulting in release of catecholamines from the adrenal medulla an increased heart rate dilatation of the coronary and muscle blood vessels and glycogen and fat mobilization as the most important features These reactions can be induced by electrical stimulation of the posterior hypothalamus, which suggests that this region is primarily involved in the control of the sympathetic reflex

A secondary adaptive reaction is mediated by the parasympathetic division which initiates the anabolic processes and restores the energy supply The endocrine system participates by a rapid secretion of ACTH ADH and probably some other pituitary hormones (growth hormone) This reaction is also fast, but has a longer latency The loss of body water is diminished protein synthesis is increased and glucocorticoids are secreted in order to build up carbohydrates to activate the reticulo-endothelial

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system and to control tissue reactions to damage Such reactions are provoked by stimulation of the anterior part of the hypothalamus

It is conceivable, therefore that the stress response is initiated by activation of hypothalamic centers through afferents from the rhinencephalic or mesencephalic limbic systems, and that the posterior and the anterior region of the hypothalamus respectively mediate the subsequent phases of the neuroendocrine emergency response Such a model would unite the ideas of Cannon on the sympathetic medullary reflex as the "emergency reaction", and of Selye on the pituitary adrenal response as the "general adaptation reaction", and would still recognize the central integrative position of the hypothalamus in these mechanisms

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The site of elaboration of LRF is probably located in a strictly circumscribed region of the hypothalamus, the so called hypophysiotropic area Pituitary transplants in this region maintain ovarian cycles (HALASZ *et al*, 1962, 1965) Also, FLAMENT DURAND (1965) showed that hypophyseal grafts implanted in this zone produced a differentiation of gonadotrophic cells This result roughly agrees with the areas in which implants of sex steroids are most effective in inhibiting gonadotrophin release

Superimposed on this regulatory mechanism, impulses can modify the secretion of LH and possibly also of FSH Some of these are inhibitory, as shown for instance by the diminished secretion of gonadotrophins observed in lactating animals, whereas others are of an excitatory nature Among the latter the impulses leading to ovulation by inducing acute discharges of LH are of prime importance It is known, mainly from the extensive work of Everett (for review, see EVERETT, 1964) that in the rat, impulses entering the anterior hypothalamus finally result in ovulation The preoptic region plays an important role as a source or channel for these impulses while lesions in this area block ovulation, electrical stimulation induces it

It is well known that the administration of central depressant drugs at appropriate times blocks ovulation, as e g pentobarbital injected at or shortly before 2 p m on the day of vaginal pro oestrus EVERETT and SAWYER (1950) showed that after this type of blockade ovulation is postponed for only 24 h, indicating that the central nervous activity responsible for ovulation has essentially a 24 hour rhythm This leads to the question why it is only at this time the so called critical period on the day of vaginal pro oestrus, that an acute release of LH occurs in the female and not on other days of the cycle

There are two possibilities either the activity of the central nervous system is inhibited on the other days of the oestrous cycle, or only on the day of vaginal pro oestrus are the impulses augmented in order to become effective It is possible that changing levels of oestrogens or even progesterone during the cycle may be responsible and one might speculate as to whether the paradoxical effect of these hormones, i e, the fact that under some circum stances they seem to promote LH release instead of inhibiting it, might cause ovulation

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REGULATION OF LH SECRETION IN THE RAT

BY

G P VAN REES

In summarizing some of the factors concerning the mechanisms by which secretion of Luteinizing Hormone (LH) is regulated in the rat, certain similarities with ACTH secretion, discussed in the previous paper by Smelik, become apparent As is the case with ACTH, a distinction can be made between mechanisms involved in maintaining a basic level of secretion and others by which LH secretion is modified acutely

The fact that secretion of LH by the anterior pituitary gland is dependent upon an intact connection with the hypothalamus is so well known that it need not be discussed any further It is also generally accepted that negative feed back mechanisms by which steroid sex hormones inhibit LH secretion and thus maintain a basic blood level of LH, are mainly the result of an action of steroids on the hypothalamus, thus altering LH secretion by a restraining influence on the amount of LH releasing Factor (LRF) which reaches the anterior pituitary gland The data concerned with this concept were obtained from experiments in which minute amounts of the steroids were implanted in different structures of the central nervous system (LISK, 1960, LISK, 1962, DAVIDSON and SAWYER, 1961, KANEMATSU, 1963) Another indication that the site of action is outside the hypophysis, is the fact that treatment with steroid sex hormones does not render the pituitary gland unresponsive to the enhancement of LH secretion caused by the administration of hypothalamic extracts containing LRF On the contrary, measurement of plasma LH in gonadectomized, oestrogen and progesterone pretreated rats has been used as an assay system for LRF (MCCANN, 1962) Also, after treatment of gonadectomized rats with high doses of synthetic steroids with an inhibitory effect on ovulation, SCHALLY and co workers (1968) did not find any decreased sensitivity to the action of a highly purified LRF preparation

tion of progesterone or oestrogen This hypothesis is supported by the fact that in female animals rendered sterile by the administration of testosterone shortly after birth progesterone has no effect in provoking ovulation although progesterone treatment could enhance LH release after electrical hypothalamic stimulation (BARRACLOUGH 1961 BARRACLOUGH and GORSKI 1961) DÖRNER and DÖCKE (1968) showed that the ovulatory action of oestrogen in immature female rats is absent after administration of testosterone shortly after birth as well as in males bearing ovarian transplants

A finding which looks disturbing at first sight is the observation that in female rats rendered sterile by placing lesions in the anterior hypothalamus progesterone may cause ovulation again (GREER 1953) However as shown by BARRACLOUGH *et al* (1964) this effect is not always present after large lesions ovulation is blocked and progesterone is ineffective The explanation for the discrepancy may be that smaller lesions do not interrupt all fibres along which the ovulatory stimuli are conducted into the hypothalamus Thus the stimuli are too weak to cause a discharge of LH However if the action of progesterone is essentially an increase of the effect of these impulses progesterone might induce ovulation in animals with partial destruction of the incoming pathways Recently complete deafferentiation of the hypothalamus has been made possible by the use of a special knife designed by HALASZ and PUPP (196) (for review see SZENTAGOTHAI *et al* 1968) It would be a most important finding if the question could be answered as to whether progesterone is still active after complete deafferentiation

One might speculate that the rhythmic impulses responsible for the induction of ovulation have essentially a 24 hour rhythm but that the efficiency of this mechanism is increased by changing blood levels of oestrogen and progesterone since blood levels of both hormones have been shown to increase before ovulation A question of importance which cannot be answered now, is the site of action of these hormones Since the preoptic region apparently plays an important role in the mechanisms controlling ovulation it would be interesting to know whether these hormones are still active in animals in which the preoptic region has been isolated from incoming fibres but with the connections with the rest of the hypothalamus left intact Another question is whether

EVERETT (1947, 1948a) showed that oestrogen, injected during pregnancy causes ovulation and when injected on day two of the 5 day oestrous cycle, advances ovulation by 24 h These experiments may be considered as a sequence to the original observation of HOHLWEG (1934) that oestrogen injected into prepubertal rats may induce ovulation and formation of corpora lutea Moreover, SWELHEIM (1965) showed that in gonadectomized female rats maintained on a low dose of oestradiol the acute administration of a high dose of the same hormone leads to elevated blood LH levels when estimated 24 h later It should be noted that this effect was not seen in male rats

Progesterone too, has the ability to promote LH release when injected on the third day of the 5 day cycle, ovulation occurs 24 h earlier (EVERETT, 1948b) In oestrogen pretreated gonadectomized females, progesterone injected at noon causes a marked increase in plasma LH levels, with maximal levels at 7 p m on the same day However, when injected at 12 p m of the same day, no increases are seen 7 h later, although the inhibitory effect of *oestrogen pretreatment is prolonged* (CALIGARIS *et al*, *1968*) This illustrates the dual effect of progesterone on LH release which with regard to the induction or inhibition of ovulation has been carefully studied by ZEILMAKER (1966) Recently, essentially the same results have been demonstrated in gonadectomized or menopausal human females with progesterone by means of immunological LH estimation methods (ODELL and SWERDLOFF, 1968) However, no effect was found in males

Thus, under certain circumstances both oestrogen and progesterone promote LH secretion The effects are seen in females but not in males Moreover while the effect of progesterone develops fairly rapidly the effect of oestrogen requires a latency period of at least some 24 h

The fact that these effects of progesterone are found *only in* females, could very well be the result of the following discrepancy between male and female animals It is known that in principle the male animal does not possess the basic mechanism of rhythmic LH discharges probably as a result of the action of endogenous androgens secreted shortly after birth (for review, see HARRIS, 1966) Therefore, it seems that this basic mechanism has to be present if one tries to induce increased LH secretion by administra

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THE ROLE OF BIOGENIC AMINES IN THE EXTRA PYRAMIDAL SYSTEM

BY

A M ERNST

1 INTRODUCTION

The motives for our investigations were derived from the following facts CARLSSON (1958) and BERTLER and ROSENGREN (1959) established that practically all dopamine in the mammalian brain is found in the extra pyramidal system (EPS), whereas noradrenaline is present there in minimal quantities, suggesting that the function of dopamine is not merely that of a precursor of noradrenaline CARLSSON (1959) had also demonstrated that reserpine could decrease the dopamine level in the EPS provoking a catatonic state in rats and a Parkinsonian syndrome in man From the earlier investigations of DE JONG (1930) and SCHALTENBRAND (1925) it was known that bulbocapnine was able to provoke a striking hypokinetic rigid syndrome (HRS), which was manifested in cats and rats as a catatonic state and in monkeys as a Parkinsonian syndrome

The results of VAN ANDEL (1959) prompted us to study dopamine in the EPS since he showed that patients with a catatonia on a chronic encephalitic basis reacted to treatment with eserine with Parkinson like phenomena, instead of the favourable reaction which he obtained in catatonic schizophrenics

2 ROLE OF CATECHOLAMINES IN THE EPS

2 1 INFLUENCE OF OCH_3-GROUPS IN DOPAMINE DERIVATIVES

First the chemical structure-effect relationship between dopamine and bulbocapnine was studied together with some other

the ovulation inducing effects of oestrogen and progesterone have a physiological significance The answers to these problems may be of utmost importance in elucidating the intricate mechanisms by which ovulation is controlled

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These results supported the hypothesis that in human patients this HRS might be created by an abnormal dopamine metabolism with O methylation at the para position, with or without O methylation at the meta position In agreement with this hypothesis was the finding that 3 4 dimethoxyphenylethylamine is present in the urine as the "Pink Spot" in schizophrenic and Parkinson patients (FRIEDHOFF and VAN WINKLE, 1963, BARBEAU *et al*, 1963)

2 2 INFLUENCE OF OH-GROUPS IN DOPAMINE DERIVATIVES

Since the catatonic action of bulbocapnine was antagonized by apomorphine (DIVRY and EVRARD, 1934) and that of dimethoxydopamine by dopa (as a precursor of dopamine) (ERNST, 1962b), a structure-effect relationship was anticipated It should be pointed out that apomorphine and the normal metabolite in the brain, dopamine, share the same basic structure, with OH groups at the phenol ring and joining an ethylamine side chain (Fig 2) In the early literature (HARNACK, 1874, AMSLER, 1923) a typical "compulsive gnawing syndrome" was described after treatment of rats with apomorphine, the presence of the corpus striatum was obligatory for this syndrome

It was decided to use this compulsive gnawing behavior in rats as a test for the structure effect relationship of apomorphine and dopamine (ERNST, 1965b) Apomorphine is a polycyclic compound

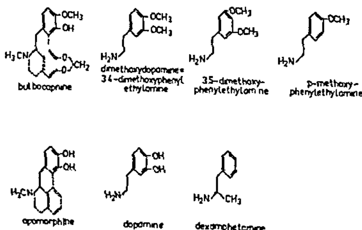


Fig 2

alkaloids of allied structure, papaverine, laudanosine, laudanine, narcotine, hydrastine, narceine and berberine (ERNST, 1962a). After administration of these compounds to cats a HRS appeared to be induced by bulbocapnine and even by papaverine, laudanine and laudanosine but never by narcotine, hydrastine, narceine or berberine. This indicated that, in order to provoke a HRS only one or two OH groups of the dopamine ring must be changed into OCH_3 groups and that the ring must have an ethylamine side chain (Fig. 1).

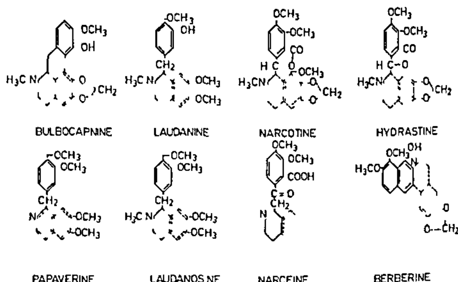


Fig. 1

This hypothesis was supported by the fact that this HRS could be antagonized by DOPA, the precursor of dopamine (ERNST, 1962b). The relationship between chemical structure and the capacity of inducing a HRS in cats was further analyzed with the aid of a number of methoxy phenylethylamine derivatives (ERNST, 1965a). From these results it was concluded that

a) only compounds having an OCH_3 group at the para position induce a HRS,
b) the presence of OH groups at the phenol ring prevents the compound from passing the blood brain barrier,
c) the presence of other OCH_3 groups next to the para position increases the duration of the HRS.

SOURKES 1964, SCHUMANN *et al*, 1963), suggesting that its action is dependent on the presence of at least one of these amines

α Methyl para tyrosine has been shown to block catecholamine synthesis without affecting serotonin levels (SPECTOR *et al*, 1965) and our results showed that the gnawing behavior after dexamphetamine was also inhibited after this pretreatment So it was concluded that the gnawing compulsion occurring after administration of dexamphetamine was caused by release of endogenous dopamine This was corroborated by the finding that pretreatment with the MAO inhibitor iproniazid enhanced the effect, since dexamphetamine itself is not destroyed by MAO, this enhancement must have been due to protection of released endogenous dopamine

The gnawing provoking action of apomorphine, however, could not be modified by pretreatment with α methyl-dopa, α methyl para tyrosine or iproniazid This strongly suggested that apomorphine does not act via dopamine release but that it has a dopamine like effect on the receptor structures We postulated that apomorphine may be capable of replacing the function of dopamine in the extra pyramidal structures for instance in Parkinson-disease where dopamine is deficient

2 4 INDIRECT ACTION OF DEXAMPHETAMINE ON LOCOMOTOR ACTIVITY

The postulation of an indirect action of dexamphetamine on gnawing behavior through the release of dopamine, was difficult to reconcile with the results of VAN ROSSUM *et al* (1962, 1964) who suggested a direct action of dexamphetamine on psycho motor activity based on their experiments with reserpine pretreatment

The decrease in the levels of dopamine, noradrenaline and serotonin caused by reserpine is well known but since, during this treatment, the normal production of the endogenous biogenic amines is continued no decisive conclusion as to a direct action of a drug can be drawn under these conditions For this reason it was thought preferable to block the production of the biogenic amines by enzyme inhibitors as in the experiments on the effect of dexamphetamine on gnawing behavior (ERNST and RAES, 1969) Rats were placed in a jiggle cage registering the psycho motor activity by amplitude and frequency changes, as a result of the movements of the animals The curves in Fig 3 and Fig 4 show a

which is capable of passing the blood brain barrier because of its lipid solubility. Dopamine, however, cannot pass this barrier so its precursor dopa which penetrates easily was used in the experiments, combined with pretreatment with the MAO inhibitor iproniazid to increase the dopamine concentration in the brain. Young Wistar rats were placed in metal cages with a wire mesh bottom. The "gnaw compulsion" syndrome was considered to be present when the rats were gnawing at the wire of the bottom for at least half a minute and when after loosening the animals from the wire, they started gnawing again.

Using this method it could be confirmed that not only apomorphine but also dopamine was able to induce a gnawing compulsion syndrome. It was also established that this gnawing effect of dopamine and apomorphine was antagonized by bulbocapnine, p-methoxyphenylethylamine, 3,4-dimethoxydopamine, but not by 3,5-dimethoxyphenylethylamine. Hence the occurrence of an OH group or an OCH_3 group at the para position of the dopamine ring appeared to be critical for the pharmacological effect. Whereas hydroxy compounds exerted an excitatory action, O-methylated derivatives had an inhibitory influence on the brain.

2.3 DIRECT OR INDIRECT ACTION ON GNAWING BEHAVIOR

Although dexamphetamine does not possess OH groups at the phenol ring it is also capable of inducing gnawing behavior (Fig. 2) (JANSSEN, 1961; RANDRUP *et al.*, 1963). The question was raised as to whether the gnawing effect of apomorphine and dexamphetamine were dependent or independent of the presence of dopamine in the corpus striatum (ERNST, 1967). Accordingly, the action of these drugs was studied in rats in which:

1. the catecholamine stores in the CNS were depleted by pretreatment with α-methyldopa or α-methyl-para-tyrosine.
2. MAO inhibition prevented metabolic degradation of the endogenous catecholamines.

From these experiments it appeared that the gnawing behavior after dexamphetamine was inhibited by α-methyldopa pretreatment, but only for 15–18 h. This indicates that the action of dexamphetamine is correlated with the recovery of dopamine and serotonin stores to normal levels (CARLSSON and LINDQVIST, 1962,

significant change of the dexamphetamine effect on locomotor activity after pretreatment with reserpine or α methyl para tyrosine Thus it appears that the increase in locomotor activity induced by dexamphetamine is effected by the release of dopamine

2 5 Sites of action of dopamine and apomorphine in the EPS

No clear information was available as to which nervous structure would be affected by dopamine or apomorphine during the com pulsive gnawing syndrome but it was likely that the corpus striatum was involved The presence of this structure appeared to be obligatory for this syndrome caused by apomorphine (Harnack 1874, Amsler 1923, Bruck, 1935), crystalline dopa (as a precursor of dopamine) or apomorphine were therefore implanted stereotaxically into different parts of the EPS of rats, after which their behavior was observed for several hours (Ernst and Smelik, 1966) Effective implants of dopa and apomorphine, resulting in an intense compulsive gnawing, appeared to be located in the dorsal part of the caudate nucleus and globus pallidum, negative results were obtained after implantation in the ventral part of the caudate nucleus in subthalamic structures and in the substantia nigra As a control meta tyrosine a drug with the same chemical structure as dopa but lacking an OH group at the para position was implanted in these places Only negative results were obtained which indicates the specificity of the dopa and apo morphine effect Thus, the site of action of dopamine as well as apomorphine appeared to be in the dorsal part of the corpus striatum The negative results of the implantation of dopa and apomorphine into the substantia nigra were somewhat disturbing since it is well known that the level of dopamine is very high in this part of the EPS However, some evidence existed that dopaminergic nerve cells lying in the substantia nigra send their fibers to the neostriatum where dopamine is released upon a o electrical stimulation (Brodal, 1963, Andén et al, 1964, Mc Lennan 1965) So we supposed that such dopaminergic nigro striatal neurons were possibly activated by other than dopami nergic stimulation Therefore physostigmine salicylate (eserine) in crystalline form was implanted stereotaxically into the substantia nigra of rats to cause a local accumulation of acetylcholine Such

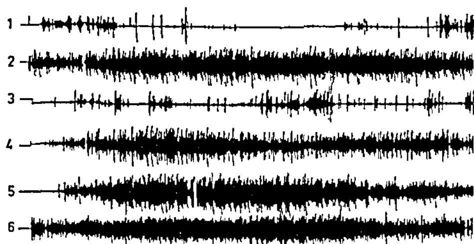


Fig 3

1 Saline	Saline	4 Reserpine	Dexamphet
2 Saline	Dexamphet	5 Reserpine	Dexamphet
3 Reserpine	Saline	6 Reserpine	Dexamphet

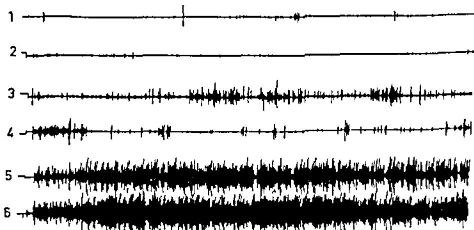


Fig 4

1 αMp Tyrosine	Dexamphet	4 Saline	Saline
2 αMp Tyrosine	Dexamphet	5 Saline	Dexamphet
3 αMp Tyrosine	Saline	6 Saline	Dexamphet

significant change of the dexamphetamine effect on locomotor activity after pretreatment with reserpine or α methyl para-tyrosine Thus it appears that the increase in locomotor activity induced by dexamphetamine is effected by the release of dopamine

2 5 SITES OF ACTION OF DOPAMINE AND APOMORPHINE IN THE EPS

No clear information was available as to which nervous structure would be affected by dopamine or apomorphine during the compulsive gnawing syndrome, but it was likely that the corpus striatum was involved The presence of this structure appeared to be obligatory for this syndrome caused by apomorphine (HARNACK, 1874, AMSLER 1923, BRUCK 1935), crystalline dopa (as a precursor of dopamine) or apomorphine were therefore implanted stereotaxically into different parts of the EPS of rats, after which their behavior was observed for several hours (ERNST and SMELIK, 1966) Effective implants of dopa and apomorphine, resulting in an intense compulsive gnawing, appeared to be located in the dorsal part of the caudate nucleus and globus pallidum, negative results were obtained after implantation in the ventral part of the caudate nucleus in subthalamic structures and in the substantia nigra As a control, meta tyrosine a drug with the same chemical structure as dopa, but lacking an OH group at the para-position was implanted in these places Only negative results were obtained, which indicates the specificity of the dopa and apo morphine effect Thus the site of action of dopamine as well as apomorphine appeared to be in the dorsal part of the corpus striatum The negative results of the implantation of dopa and apomorphine into the substantia nigra were somewhat disturbing, since, it is well known that the level of dopamine is very high in this part of the EPS However, some evidence existed that dopaminergic nerve cells lying in the substantia nigra send their fibers to the neostriatum where dopamine is released upon a o electrical stimulation (BRODAL, 1963, ANDÉN *et al*, 1964, MC LENNAN, 1965) So we supposed that such dopaminergic nigro striatal neurons were possibly activated by other than dopami nergic stimulation Therefore physostigmine salicylate (eserine) in crystalline form was implanted stereotaxically into the substantia nigra of rats to cause a local accumulation of acetylcholine Such

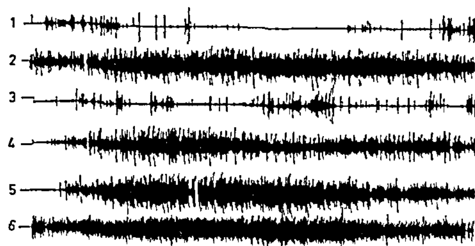


Fig 3

1 Saline	Saline	4 Reserpine	Dexamphet
2 Saline	Dexamphet	5 Reserpine	Dexamphet
3 Reserpine	Saline	6 Reserpine	Dexamphet

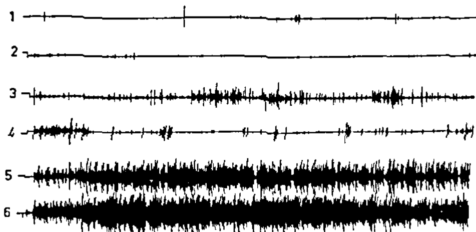


Fig 4

1 αMp Tyrosine	Dexamphet	4 Saline	Saline
2 αMp Tyrosine	Dexamphet	5 Saline	Dexamphet
3 αMp Tyrosine	Saline	6 Saline	Dexamphet

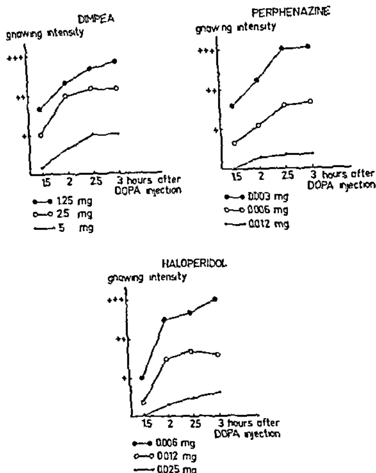


Fig 5

For practical purposes apomorphine was used in further experiments since the same receptor is involved for dopamine as well as apomorphine (ERNST, 1967, and confirmed by ANDÉN, 1967) Apomorphine induced gnawing behavior appeared to be blocked as well, and a parallel shift was present in the dose response curves of the three compounds A reasonable blockade of the apomorphine effect was also obtained by a minimal dose of perphenazine and this equilibrium of doses could be used as a "Unit" When the rats were treated with an increasing number of "Units" (Fig 6), the

an implant caused compulsive gnawing behavior, which was inhibited by pretreatment of the rats with atropine. Implantation of eserine into the caudate nucleus or the globus pallidum was ineffective (SMELIK and ERNST, 1966). Hence it was concluded that cholinergic nerve fibers end synaptically on the dopaminergic nigra cells. Since cholinergic stimulation of these cells provoked gnawing behavior, a functional significance of this nigro-neostriatal dopaminergic tract was demonstrated for the first time.

2.6 CENTRAL DOPAMINERGIC RECEPTORS

It appeared that these central dopaminergic receptors (CDR) in the corpus striatum are very specific. Phenoxybenzamine, a potent and centrally acting α-adrenergic receptor blocking agent, was not able to prevent or to antagonize compulsive gnawing, nor did nethalide, a β-adrenergic receptor blocking agent. Pretreatment with anti-histamine and anti-serotonin drugs could not prevent the syndrome, nor did high doses of atropine. Accordingly the CDR involved in compulsive gnawing behavior are different from known autonomic receptor structures.

As we know from earlier experiments (ERNST, 1962a, b) dimethoxyphenylethylamine (= dimethoxydopamine = DIMPEA) and some other drugs chemically related to dopamine can produce a hypokinetic rigid syndrome in animals, which was confirmed by BARBEAU (1966). This hypokinetic state and other Parkinsonian symptoms are also provoked in many patients treated with neuroleptic drugs such as chlorpromazine, perphenazine, haloperidol etc. The studies of BARBEAU (1967) and DA PRADA and PLETSCHER (1966a, b) suggested that these Parkinsonian symptoms are caused by CDR blokkade. Stimulated by the work of VAN ROSSUM (1966) who was able to block the peripheral depressor effect of dopamine receptors by neuroleptic drugs, we again used the compulsive gnawing syndrome in rats as a model to provide more evidence for a specific CDR blockade by these neuroleptic drugs.

Rats pretreated with iproniazid were injected with different doses of perphenazine, haloperidol or DIMPEA; all animals were treated one hour later with L-dopa, after which the intensity of the gnawing was measured. It appeared that all three substances were able to block the gnawing behavior and moreover these drugs caused a parallel shift in the dose response curves (Fig. 5).

on the peripheral receptors of the intestine The catatonia produced by tryptamine should then be regarded as a blockade of the 5 HT receptors It was also established (VAN ANDEL and ERNST 1961) that the tryptamine catatonia could be intensified by atropine in moderate doses and completely abolished by physostigmine salicylate (eserine) These results led to the hypothesis that tryptamine can block (or compete with) 5 HT receptors thus eliminating a function which tends to potentiate certain acetylcholine activities in the CNS and that conversely a hypocholinergic function would produce catatonia

VAN ANDEL (1959) investigated catatonia in 18 clinical patients all showing hypokinesia mutism and stupor Without knowing the clinical diagnosis (schizophrenic hysterical or encephalitic patients) he treated them with eserine injections to elevate the concentration of acetylcholine in the CNS Fourteen patients out of the 18 reacted favourably they started to talk and to laugh and their contact with persons in their environment was much better One patient of the group of 18 did not show any improvement and 3 patients reacted in quite a different way they showed Parkinson like symptoms after the eserine injection Later on it appeared from the clinical diagnosis that the 14 patients who reacted favourably were all schizophrenics the patient who did not react was a hysterical one and the 3 who showed an aggravation of their symptoms and Parkinson like phenomena were catatonics on a chronic encephalitic basis Thus our hypothesis is justified in schizophrenic patients but not in chronic encephalitic ones This led us to the idea that yet another chemical mediator might be involved in catatonia phenomena of these latter patients which prompted our study on dopamine in the FPS

3 1 SITES OF ACTION OF 5 HT IN THE EPS

To determine more exactly the role of 5 HT in the EPS rats were injected with 5 HTP after a pretreatment with the MAO inhibitor iproniazid The animals first showed tremor and stereotyped movements of the head and forelegs they moved around the cages and after some time they started to gnaw

This gnawing behavior was not similar to the dopamine and apomorphine gnawing (ERNST 1965b) since the animals took the wire of the floor and held it convulsively, without moving around

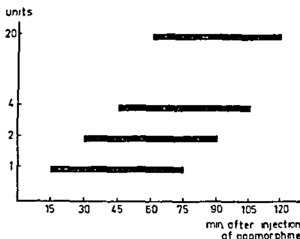


Fig. 6.
1 unit = 0.025 mg perphenazine followed one hour later by 0.5 mg apomorphine

gnawing *intensity* and the *duration* of the gnawing period, was the same in all animals, only a parallel shift in the starting point of the syndrome was observed. Thus the three compounds gave a parallel shift in the dose response curves, without affecting the maximum that could be obtained. This would suggest a competitive type of antagonism on the CDR.

3. ROLE OF SEROTONIN (5-HT) IN EPS

In the introduction results suggesting an important role of 5-HT in the extrapyramidal system (EPS) were mentioned but most of this work was unknown when we started our research on this subject. It struck us at that time (ERNST *et al.*, 1961) that VAN NIEUWENHUYZEN (1936) had been able to provoke a catatonic state in cats by tryptamine, a compound which lacks only one OH group on the 5 position of 5-HT. Since 5-HT is not able to pass the blood-brain barrier, cats were given tryptamine injections into the cysterna magna, producing a catatonic state, and this catatonia could be antagonized by 5-HT. These central effects on the autonomic nervous system of 5-HT and tryptamine were qualitatively of the same order, but the effect of tryptamine was much weaker. These facts led to the hypothesis that both drugs were acting on the same receptor in the CNS, just as GADDUM (1953) demonstrated

SUMMARY OF THE RESULTS AND A HYPOTHESIS

Only compounds having an OCH_3 group at the para position of the phenol ring joining an ethylamine side-chain are able to provoke a HRS, by blocking the dopamine receptors in the EPS This blocking effect seems to be obtained by a competitive antagonism The presence of more OCH_3 groups next to the para position, increases the duration of this HRS

The presence of OH groups in this configuration prevents the compounds from passing the blood brain barrier But amino acids bearing no net charge and polycyclic lipid soluble compounds rapidly pass this barrier, although they possess OH groups at the para and meta position of the ring They are able to mimic the effect of dopamine in the EPS by utilizing the dopamine receptors

With compounds in which OH and OCH_3 groups are absent from the basic chemical structure, there is a good chance that their action is an indirect one, by dopamine release

Cholinergic nerve fibers end synaptically on the dopaminergic nigra cells, hence cholinergic stimulation causes dopamine production in the corpus striatum via the nigro neostriatal dopaminergic fibers

On the basis of these results the following hypothesis is proposed In patients with Parkinson s disease based on a degeneration of the substantia nigra including the dopaminergic cells a comparative surplus production of acetylcholine may exist in the area of the EPS In patients showing a Parkinson like syndrome, as seen after treatment with neuroleptics of the phenothiazine or reserpine type this is caused respectively by the dopamine receptors in the corpus striatum being blocked or by the dopamine stores being depleted Therefore, it should be possible in addition to the usual treatment with anticholinergic drugs to use dopaminergic agents therapeutically, which rapidly cross the blood brain barrier and mimic the dopamine effect Apomorphine would meet these requirements, but its emetic effect is a serious objection

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We also performed implantation studies with 5 HT or 5 HTP in different parts of the EPS, with or without pretreatment with iproniazid (HADŽOVIĆ and ERNST, 1968) It was found that 5 HT induced gnawing behavior only when implanted in the rostro ventral part of the corpus striatum (Fig 7) In all other parts of the corpus striatum 5 HT or 5 HTP induced neither gnawing nor tremor, even when implanted into the area where the implantation of dopa or apomorphine did provoke gnawing behavior (ERNST and SMELIK, 1966) Tremor was induced by implantation of 5 HT or 5 HTP into the substantia nigra, both with and without iproniazid pretreatment

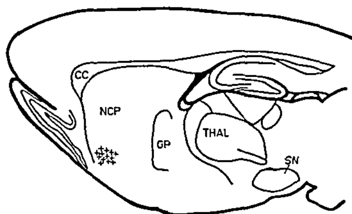


Fig 7

To confirm these findings, bilateral lesions were made by high frequency cauterization in the rostro ventral part of the corpus striatum, and 5 days later 5 HTP, L dopa or apomorphine was administered i p (with iproniazid pretreatment) In these animals, 5-HTP induced only tremor followed by stereotyped movements of the head and forlegs, while gnawing behavior was provoked only after administration of L dopa or apomorphine These findings clearly demonstrated that there are two separate sites of action of 5 HT a site of action for gnawing behavior, located in the rostro ventral part of the corpus striatum, an for the tremor in the substantia nigra Possibly there is also a third site of action provoking the stereotyped movements

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SOME RECENT DEVELOPMENTS IN CLINICAL NEUROPHYSIOLOGY

THE RECORDING OF AVERAGE VISUAL RESPONSES AND THE DETERMINATION OF REGIONAL CEREBRAL BLOOD FLOW

BY

O MAGNUS

1 INTRODUCTION

Clinical neurophysiology is perhaps as old as neurology The eliciting of a kneejerk or of a pupillary reflex is as much a clinical neurophysiological method of investigation as is the determination of the conduction velocity of a nerve or the recording of an average cortical response to photic stimulation The tonic neck reflexes described by MAGNUS and DE KLEIJN (1912 1924) which are part of the neurological examination in patients with severe brain damage are a good example of the development of a neurological diagnostic method from pure neurophysiological research Conversely clinical neurology has contributed much to neurophysiology In this connection one can mention the neurologist HUGHLINGS JACKSON (1931) and the neurosurgeon Penfield who by careful observation of epileptic seizures and the correlation of their observations with the pathological regions from which these seizures originated contributed so much to our knowledge of the functional localisation in the cerebral cortex (PENFIELD and JASPER 1954) Moreover such observations have led to extensive and very rewarding experimental neurophysiological research

Of course the aims of clinical neurophysiology are fundamentally different from those of experimental neurophysiology The first responsibility of the clinical neurophysiologist is towards his patient But modern technical developments offer to the clinical neurophysiologist methods which enable him to obtain data from patients which can also be of value to neurophysiology It is of

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A number of methods of automatic quantification have been introduced For a discussion of these methods I should like to refer to a report on the symposium on this subject by STORM VAN LEEUWEN and MAGNUS (1961) The introduction of special purpose computers has made it possible to record in man average cortical responses to various stimuli The results of some investigations on the recording of the average visual responses in normal subjects and patients will be discussed in the first part of this paper

3 A third limitation to the improvement of correlations between EEG and brain disturbances is due to the fact that detailed information concerning the nature and the extent of a cerebral lesion is often lacking at the time the EEG is recorded Originally such information was only based on the clinical neurological examination and in some cases on findings at operation or at autopsy However parallel with the EEG a number of other diagnostic methods have been developed such as various neuroradiological contrast methods echo-encephalography and the various types of scintigraphy Within the domain of clinical neurophysiology the measurement of the cerebral circulation with the aid of radio active isotopes appears to be of importance Results obtained with this method are the subject of the second part of this contribution

2 THE AVERAGE VISUAL CORTICAL RESPONSE

The influence of rhythmic photic stimulation on the EEG has already been studied by ADRIAN and MATTHEWS (1934) It was introduced into routine clinical EEG i a in order to elicit epileptic activity by V and W GREY WALTER in 1949 In occipital areas responses were also found to isolated flashes but because of their low amplitude it was often difficult or hardly possible to distinguish them from the ongoing activities This was improved by the method of photographic superimposition (DAWSON 1947) With this method Ciganek studied the visual responses in 75 normal adults But only the introduction of special purpose computers has made it possible to study these responses in a clinical setting on a larger scale For this purpose it is necessary to add a relatively large number of responses in order to eliminate the spontaneous activity

The investigations to be reported here have been carried out with a 16 channel van Gogh electroencephalograph a Mnemotron

particular importance that some of these methods are not harmful and often not even cumbersome to the patient and that therefore some of them can also be applied to normal subjects

The most important method of clinical neurophysiology is electroencephalography Since the discovery of the electroencephalogram of man by Berger 40 years ago the diagnostic value of the EEG has been improved continuously by making empirical correlations between EEG patterns and brain disturbances

However, there are limitations to the further improvement of these correlations, three of them will be mentioned here

1 The EEG can only record electrical phenomena which are caused by synchronous potential variations of large neuron populations in the cerebral cortex In other words activities of small groups of neurons which for instance, give rise to finger movements or elementary local sensations are not seen in the EEG COOPER *et al* (1965) and more recently STORM VAN LEEUWEN (1968) in his inaugural address have drawn attention to this fact

For these reasons only those pathological processes which give rise to synchronous changes of the electrical activities of relatively large cortical areas manifest themselves in the EEG The most important of these processes are

1 Disturbances of metabolism either diffuse (anoxia hypoglycaemia or intoxication) or else local (ischaemic lesions tumors etc)
2 Alterations of the level of consciousness
3 Epileptic discharges which are nearly always associated with abnormal synchronisation of electrical activities of neurons

In this connection it should be stressed that the EEG derives its main importance from the fact that it detects fundamentally different aspects of pathological brain disturbances from other methods of neurological examination

2 A second limitation is due to the fact that the EEG cannot easily be quantified and therefore there tends to be a subjective factor in the evaluation of a particular record This can be diminished to a certain extent by giving an objective description and a functional interpretation of the EEG without knowledge of the clinical findings Only after this has been done should the EEG data be correlated with the clinical data

1 In normal subjects as well as in patients the average visual responses (AVR) were reproducible

2 The findings of Ciganek that the response consists of an occipital primary part during the first 125 msec after the flash, a somewhat more diffuse secondary part for 125–250 msec after the flash followed by a rhythmic component of approximately the frequency of the α rhythm have been confirmed (Fig 1)

3 Even in normal subjects there are large interindividual differences in the configuration of the response But in a single normal subject the responses are symmetric within narrow well definable limits In this respect the average visual response is similar to the EEG

4 The configuration of the response is strongly influenced by changes of the level of consciousness During drowsiness and sleep marked changes occur in the shape of the AVR and in the latencies of its components (Fig 2) In this respect again the AVR behaves like the EEG, perhaps it is even more sensitive

5 In older subjects the response often has a different shape from that in young adults As has been shown already by Ciganek there is a surface negative spike with a peak latency of 80 msec in the occipital region However, it appears that this component is only found constantly in young adults With increasing age a surface positive spike with the same latency and location is found more frequently

6 In accordance with data from the literature a *pathological* asymmetry of the AVR has been found in 21 out of 22 patients with a considerable contralateral defect of the visual field In the only exception, the asymmetry just failed to reach the previously set limits of normality However also in the majority of patients with lesions in one hemisphere, which did not interfere with the visual pathways, an abnormal visual response [illegible]

[illegible]

In lesions interfering with the visual pathways or affecting the occipital region there was always an abnormal asymmetry of both the primary and the secondary part of the AVR When a lesion outside the visual pathways gave rise to an abnormal asymmetry

Computer of Average Transients (CAT), an XY plotter and a FR 1300 Ampex tape recorder, which made it possible to store the electrical activity of 13 channels simultaneously and to determine the average responses partly on line and partly afterwards from the tape In this way, the potential distribution of the various components of the average responses could be determined Jonkman and Ponsen studied the average responses to light flashes in 16 young and 10 older normal subjects and in 130 patients, mostly with neurological disorders Only the main conclusions of these investigations will be mentioned They are described in detail in the thesis of JONKMAN (1967)

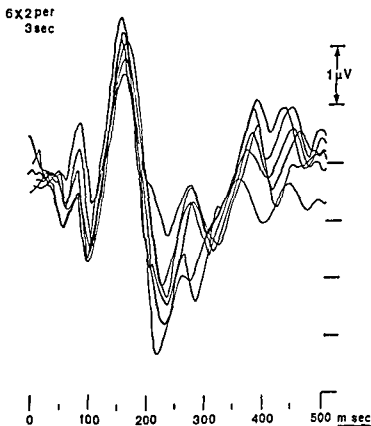


Fig 1
6 Superimposed average responses to light flashes recorded in the left occipital to ear lead Each trace in the average of 50 responses Flash frequency 2 per 3 sec A primary part in the first 100 msec, a secondary part between 100 and 225 msec after the flash and a rhythmic component can be distinguished Electrode positions in Figs 1–8 corresponding with "10–20 system"

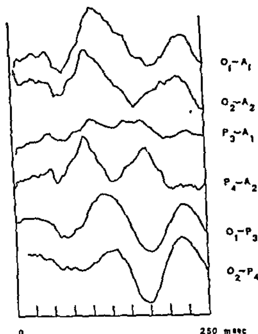


Fig 3

AVR recorded in a man, age 63, with a vascular lesion in the left parietal region There was no hemianopsia The asymmetry is most marked in the parietal region (JONKMAN, 1967)

shown that there are a number of parallels between the AVR and the ordinary EEG At first sight it would seem unlikely that unilateral lesions not affecting the visual system can give rise to an abnormal asymmetry of the AVR However, in this respect also there is correspondence with the EEG Local pathological abnormalities in the EEG consisting of delta waves or spike discharges are usually not associated with a disturbance of the function of the corresponding part of the cerebral cortex For the time being it is not possible to indicate what must be the nature, location and extent of a lesion in order to give rise to a pathological asymmetry of the AVR Perhaps experimental neurophysiological research could provide interesting data on this problem

So far, the recording of average responses to light flashes has not yet provided an essential contribution to neurological diagnosis This appears to be due partly to the fact that the abnormalities



Fig 2

Primary part of the AVR Same derivation
——— subject awake
- - - - during sleep (JONKMAN, 1967)

of the AVR, this proved to be associated with an asymmetry of the primary part of the response and not with an asymmetry of the secondary part These findings have been confirmed by OOSTERHUIS *et al* (1969), who studied the AVR in 30 patients with cerebro-vascular lesions

Investigation of the average visual response to light flashes has

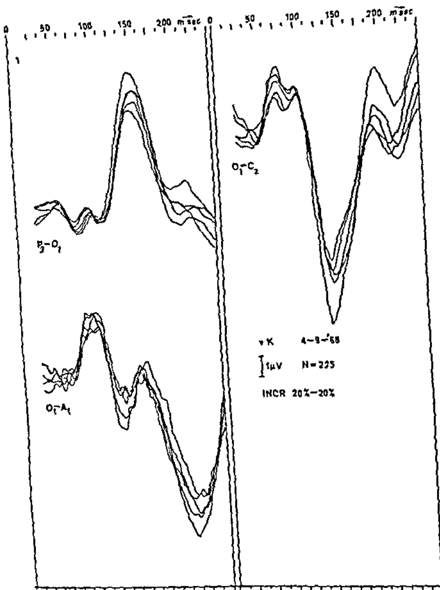

0
50
100
150
200 msec
0
50
100
150
200 msec
1
P3-O1
O1-A1
O1-C2
vK 4-9-'68
1µV N=225
INCR 20%-20%

Fig 4a.

of the AVR are not specific for the type or location of a lesion Only with some vascular lesions was the response disturbed particularly in the area of the lesion (Fig 3) Finally it must be stressed that the method is expensive and time consuming and that it must be carried out by an experienced investigator

Further quantification of the stimulus parameters appeared to be necessary Following earlier investigations by DE LANGE (1958) in which modulated light was used to study flicker fusion, VAN DER TWEEL (1961) introduced sinusoidal modulation of light intensity as a stimulus for the recording of evoked potentials With this type of stimulus it is possible to study the relationship between stimulus and response in a quantitative way The average light intensity remains unchanged with different frequencies and modulation depth Moreover the variations in light intensity correspond more with those of physiological stimuli The investigations of VAN DER TWEEL and SPEKREIJSE (1965, 1966) with this type of stimulation have shown that the complicated responses are caused by different mechanisms which can be distinguished and separated Moreover some idea was obtained of the sequence in which these mechanisms occur The quantitative data which have been obtained appear to clarify how fast phenomena are attenuated in early phases of the visual system

With the same method Storm van Leeuwen and Kamphuizen and Mol have obtained interesting results in neurological patients (vide VAN DER TWEEL *et al*, 1967) In our unit Ponsen studied the average responses to step variations of a constant average light intensity, the modulation depth as well as the average light intensity could be changed in different experiments The responses to increase and decrease of light intensity were studied separately In order to obtain reproducible results it proved to be important that the subject remained well adapted, that there was no change in the background light, and that the subject remained awake with eyes open When these conditions were fulfilled reproducible responses were obtained (Fig 4) This was even true for a modulation depth of 2½ %, a variation in light intensity which could just be perceived, it also applied to different derivations The average responses to decrease of light intensity were similar to those obtained with increase of light intensity, but the amplitudes were usually lower The average responses obtained in different test

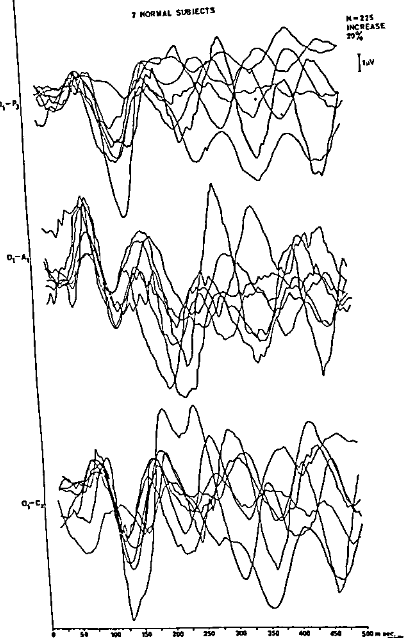


Fig 5
AVR to increase in L I recorded in 7 normal young adult subjects superimposed Modulation depth: 20 %

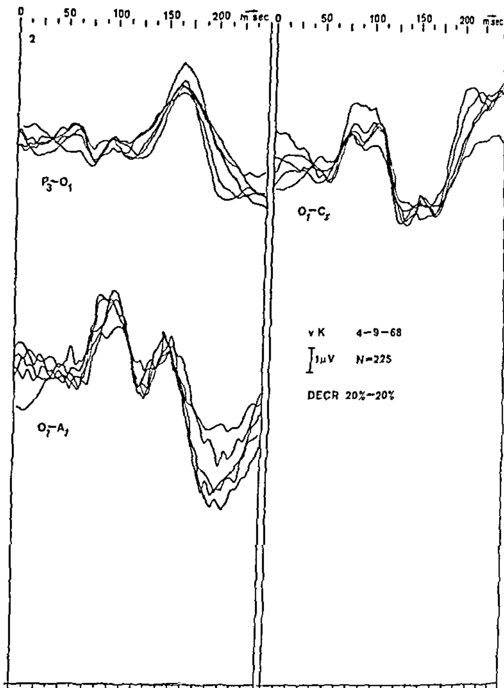


Fig 4b

Fig 4

Normal subject (♂, 20 yrs) 5 Superimposed average responses to step variations of light intensity (L I) *Modulation depth 20 % Intervals* between increase and decrease of L I 1 sec Each individual curve re presents *the average of 225 responses*

a Responses to increase of L I

b Responses to decrease of L I

subjects were also similar at least for certain modulation depths (Fig 5)

Also with this stimulus modality the responses were very sensitive to variations in vigilance or consciousness They changed in configuration and latency with drowsiness and sleep (Fig 6) When the test subjects remained awake during the procedure, changes in

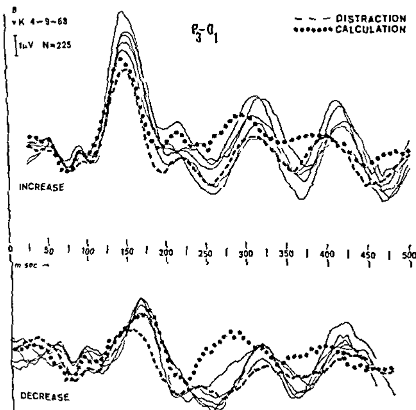


Fig 7

AVP, recorded in the same subject as in Fig 4
——— Eyes open counting flashes
During conversation
During calculations
There are only minor differences which are most marked in the rhythmic component Note that this component is more marked after increase than after decrease of the L.I

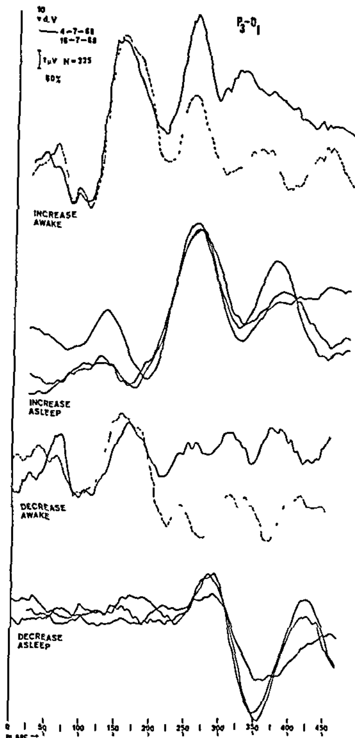


Fig 6

AVR to increase and decrease of L I recorded in a normal young adult subject Modulation depth 60 %

Note marked difference between responses recorded during sleep as compared with recording whilst the subject was awake

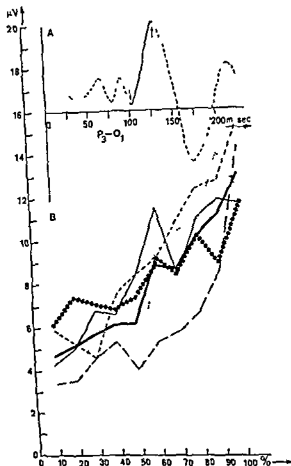


Fig 8.

Graphs representing the relation between the modulation depth and the amplitude of one gradient of the AVR in 5 normal young adults

Upper part The drawn section of the AVR indicates the gradient, the amplitude of which was measured

Lower part Each graph represents the average of 2 separate experiments. For each modulation depth the average of 225 responses to increase of L I. was determined The heavy drawn line represents the average of the 5 other graphs

the degree of vigilance caused by *different tasks only* gave rise to slight modifications of the average response (Fig 7)

The relation between the modulation depth and the amplitude of the main component of the average response varied from subject to subject Fig 8 shows this relation for 5 subjects, together with the average for these subjects as recorded in the parieto occipital derivation

So far the investigation has been limited to 8 young normal adults When the method has been sufficiently standardised and the normal variations are known the investigations will be extended *to neurological patients*

The question arises as to which direction these methods of investigation should be developed

Of course it is not possible to predict which new methods will become available to electro neurophysiology in the near future At present it seems justified to state that automatic analysis of certain aspects of the spontaneous electroencephalogram or of evoked potentials will provide information mainly on those disturbances of the brain which lead to either local or else diffuse synchronized changes of the electrical activity of the cortex

These *disturbances have been mentioned above*

Recent developments seem to point in the same direction In this connection the Contingent Negative Variation (CNV) or "Expectancy Wave" discovered by GREY WALTER and colleagues (1964a b) should be mentioned They demonstrated that preceding an expected signal a slow diffuse negative potential occurs with a maximum at the vertex, particularly when the signal is to be followed by an action This *CNV* can be "conditioned" or "deconditioned" in other words it is a suitable sign to study a certain psychological attitude which Grey Walter has called "expectancy" The type of situation which leads to this expectancy does not manifest itself in the CNV The investigation of this phenomenon seems to open perspectives for psychological and psychiatric research in which the CNV can serve as a monitor Investigations by Storm van Leeuwen on the correlation of certain types of electrical activity of the cortex and of subcortical structures with certain behaviour patterns of dogs point in the same direction *The results* of some of these investigations will be described by Storm van Leeuwen in the subsequent communication

angiography RISA is a macro molecular substance which, during the period of measurement remains almost entirely within the blood vessels The gamma radiation is measured with the aid of a scintillation detector over the parieto temporal region and is plotted on paper The curve obtained shows a steep rise, a plateau and an exponential decrease (Fig 9) Therefore when plotted on semilogarithmic paper the phase of decline of the radio activity is a straight line The time in which the radio activity decreases to half of its maximal value has been called by van den Berg the Circulation Time, Descending phase ½ (CTD½) It is a fair measure for the clearance of the RISA from the intracerebral blood pool With this method van den Berg studied approximately 200 patients in whom carotid angiography was performed and established criteria for normality and abnormality, by correlating the CTD½ with the type and extent of the lesion Then with VAN DER DRIFT and VAN DEN BERG (1967) we made a comparison between the EEG findings and the CTD½ (Table 1) In general, there proved to *be a good correlation between the severity of the EEG abnormalities* and the degree of slowing of the cerebral circulation However, there were a number of interesting exceptions There was a small number of cases with marked EEG abnormalities and a normal cerebral circulation In particular there were 4 cases of encephalitis

TABLE 1

EEG type	CTD½ in sec			
	≦ 3 0	3 1–3 8	3 9–5 0	> 5 0
I	0	58	42	10
II	5	11	143	5
III	4	0	0	66

Correlation between degree of EEG abnormalities and cerebral blood flow as determined by intra carotid injection of RISA

CTD½ 2½–3 sec normal
3 1–3 8 sec mildly abnormal
3 9–5 0 sec moderately abnormal
> 5 0 sec severely abnormal

EEG type I no or mild abnormalities
EEG type II marked unilateral abnormalities
EEG type III severe disturbances on one side with less marked abnormalities on the other side

As has already been stated the application of various methods of automatic analysis of the EEG has so far had little practical importance for neurological diagnosis. Therefore other clinical neurophysiological methods which can be applied to neurological patients are particularly welcome. In the following section some investigations on the cerebral circulation will be briefly discussed.

3 THE MEASUREMENT OF THE CEREBRAL CIRCULATION WITH RADIO-ACTIVE ISOTOPES

Parallel to studies of FAZIO and colleagues (1962, 1963) VAN DEN BERG (1965) and VAN DEN BERG and VAN DER DRIFT (1962) used radio active iodine linked to human serum albumin (RISA). This substance was injected into the carotid artery following

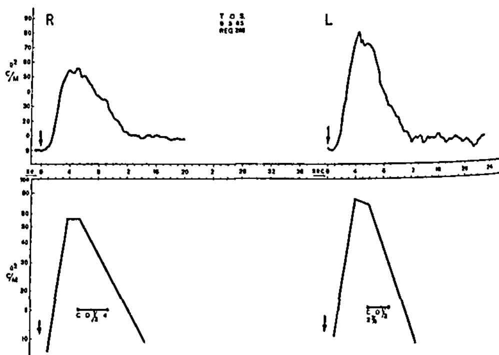


Fig 9

♀ 44 yrs with a deep tumor in the right parietal region

Upper part RISA curves from right and left parieto temporal region

Lower part The same curves represented on a semi logarithmic scale. CTD½ = 4 sec on the right side, 2¾ sec on the left side (normal values 2½–3 sec)

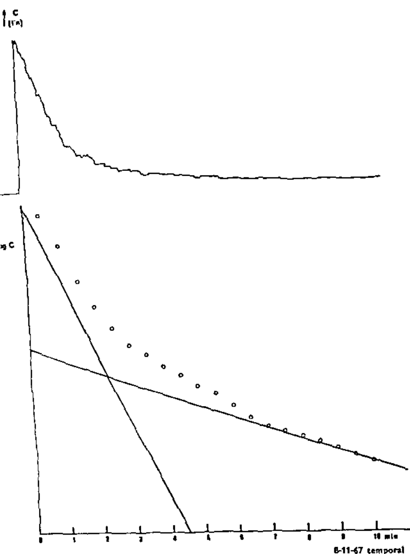


Fig 10

♂ 66 yrs Slight Rt hemiparesis and hypersensitivity of Lt carotid sinus Lt carotid angiogram and cerebral blood flow as determined with Kr 85 normal

Upper part Curve obtained after intra carotid injection of Kr 85
Lower part The same curve represented on a semilogarithmic scale. This curve can be resolved into 2 mono exponential components (steep line = fast component other line = slow component) Fast 95 cc/100 g/min, slow 20 cc/100 g/min, two comp 61 cc/100 g/min, non comp 59 cc/100 g/min

in whom intoxication and disturbances of metabolism which are not directly related to the circulation were considered to be responsible for the EEG abnormalities In these cases the EEG provided better information concerning the cerebral disturbances than the measurement of the circulation

On the other hand there was a larger group of patients in whom the EEG showed little or no abnormalities but in whom there was a marked slowing of the cerebral circulation This group included mainly patients with a primary chronic vascular insufficiency and patients in the chronic stage after a severe head injury In these cases we have assumed that the brain and particularly *the cortex had adapted to the diminished circulation* probably mainly by atrophy This could be demonstrated in some cases by pneumo encephalography

In this group of patients the measurement of the circulation provided a better picture of the cerebral disorder than did the EEG

LASSEN and colleagues (1963) have introduced the measurement of the regional cerebral bloodflow in man with the aid of Krypton 85 or Xenon 133 which diffuse directly into the brain tissue When one measures the radio activity in a corresponding way a curve is obtained which after a rapid increase shows a gradual decrease extending over minutes instead of seconds as is the case with RISA The descending phase can usually be considered to consist of 2 exponential components (Fig 10) a fast and a slow component The first one is considered to be mainly a measure for the bloodflow in the grey matter the second one for the bloodflow in the white matter This method is likely to give a better picture of the supply of the inert gas *at the level of the ganglion cells*

At first Krypton 85 was used which emits mainly beta rays and only a small percentage of relatively hard gamma rays More recently Xenon 133 has been introduced which has the advantage of emitting almost exclusively soft gamma rays which makes it possible to measure more locally than with Krypton A drawback of Xenon 133 is that its half life is only 5 days in contrast with 12 years for Krypton 85

Both gases are exhaled directly through the lungs so that there is very little recirculation and the radiation dose is completely harmless

In our hospital Krypton 85 has been used by JONKMAN VAN DEN

the tumor itself, can give rise to a shunting of blood to the veins (intra cerebral steal) This can sometimes be demonstrated with cerebral angiography (FEINDEL and PEROT, 1965) In the curves obtained with Xenon 133 in such cases there is a high initial peak, similar to the one obtained in cases of arterio venous aneurysm (Fig 11 VAN DER DRIFT *et al* 1968)

It is hoped and expected that the application of this method of investigation will not only be useful for diagnosis but that it will

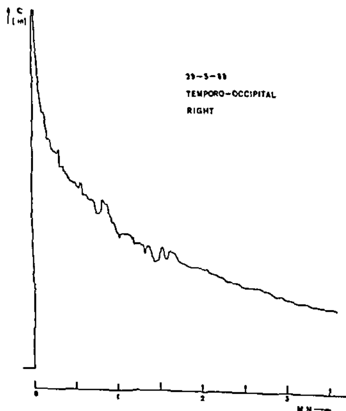


Fig 11

♂ age 59 with a Rt temporo-occipital astrocytoma grade IV Radio activity recorded in the region of the tumor during initial period of 3 min after intracarot d
e high initial peak indicating the
ed in the carotid angiogram by
the tumor (cf VAN DER DRIFT
et al, 1968)

BERG and VAN DER DRIFT (1968) 52 times for the measurement of the cerebral circulation in patients with various brain disorders So far there has been no clear correlation between the location of a lesion and values obtained *for the different components of* the circulation curve To a certain extent this could be explained by the fact that some lesions such as brain tumors for instance can *either be associated with an increase or with a decrease of the* regional cerebral bloodflow However, it could be shown that in patients with focal lesions there are larger regional differences than in patients with diffuse disturbances These differences were relatively small In cases of a unilateral space occupying lesion giving rise to a substantial displacement over the midline as shown by echo encephalography and/or carotid angiography the average values for the circulation were lower than in the group of cases without a displacement This can be explained by the fact that the displacement itself gives rise to a disturbance of the circulation

So far we have studied the circulation in 6 patients with Xenon 133 The preliminary results confirm the findings of HOEDT RASMUSSEN (1967) that with this isotope one can measure more locally and obtain a better impression of the regional differences in cerebral bloodflow

It is possible to record simultaneously with 16 channels over part of one hemisphere In this way valuable information has been obtained on the flow in the region of acute cerebro vascular lesions (HOEDT RASMUSSEN *et al* 1967) It appears that there is often maximal dilatation of capillaries owing to vaso motor paralysis The auto regulation of these vessels is lost and the blood flow depends entirely on the difference between the arterial and venous pressure i e on the bloodpressure Vaso dilator drugs have therefore *no influence on the blood vessels in the* affected area and when they cause a fall in blood pressure they may even have an adverse effect on the circulation in the ischaemic lesion

As the uptake of oxygen by the damaged tissue is diminished oxygenated red blood may reach the veins even if the local bloodflow is normal or subnormal This has been called 'luxury *perfusion* by LASSEN *(1966)*

The method described can also provide interesting information on disturbances of circulation caused by brain tumors

Vaso motor paralysis but also the abnormal vascularisation of

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provide us with better information on the pathophysiology and pharmacology of the brain circulation There is no strict parallelism between the results of the measurement with RISA and those obtained with Krypton 85 or Xenon 133 A comparison between the results of these 2 methods should provide additional information

Correlation of these findings with the EEG recorded at the same time should furnish interesting results concerning those EEG abnormalities which are caused by *disturbances of the circulation*

SUMMARY

Some limitations to the improvement of the contribution the EEG can make to the neurological diagnosis are discussed The main factors appear to be

1 Only those brain disturbances which give rise to abnormality of synchronised activities of *large neuron populations of the cortex can be* recorded
2 The EEG data do not lend themselves easily to quantification
3 At the time the EEG is recorded there is often insufficient information on the exact type location and extent of a brain lesion

In the first part investigations concerning the average visual response to lightflashes in 26 normal subjects and 130 neurological patients are discussed

Some findings on average responses to step variations of a constant average light intensity in normal subjects are reported Responses to increase and decrease of light intensity have been described separately and compared The correlation between the modulation depth and the amplitude of the main component of the response has been studied Problems concerning the application of these methods to clinical neurology are discussed

In the second part investigations concerning the cerebral circulation with the aid of intra carotid injection of RISA Krypton 85 and Xenon 133 are reported The correlation between the CTD½ which is a measure of the *clearance of RISA from the cerebral bloodpool and the degree* of abnormality of the EEG recorded in the same period, has been studied The categories of patients in which there appears to be a discrepancy between the EEG and the cerebral circulation, as studied by RISA, are briefly discussed

The importance of the application of different clinical neurophysiological methods and of the integration of the results is stressed

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ELECTRICAL ACTIVITIES OF THE BRAIN AND BEHAVIOUR

BY

W STORM VAN LEEUWEN

One of the first people to make an attempt at a systematic detailed investigation of the nervous system was Sherrington He restricted his investigations to a system which is, or appears to be more simple than the brain, namely the spinal cord This is not the place to enumerate the beautiful experiments which led him finally to his concept of the integrative action of the central nervous system For the present it will suffice to remember that Sherrington understood very well the interaction taking place between cells Though his model was simple, for he was thinking in comparatively small numbers rather than in large masses of cells, it nevertheless remains essentially valid Its importance is the influence one cell has on another outside the direct transmission of action potentials Sherrington's central *excitatory* state and central inhibitory state are still valid concepts In *Sherrington's time* the relation between these states and electrical phenomena had *not* been proved, but obviously his states are directly comparable to the present concepts of excitatory and inhibitory synaptic potentials The main significance of the *concept is that at the* synaptic level a process takes place which does *not follow the all* or none characteristics of the impulse transmitted along the *neuron*, but which is comparable to an analogue system, *gradually* accumulating or decreasing as excitatory or inhibitory *processes come in*

Magnus extended the work of Sherrington by including the brain stem in his investigations Thus rather than investigating only the spinal reflexes as Sherrington did, Magnus investigated the more complex mechanisms involved in the maintenance of posture Magnus's collaborators Rademaker and De Kleyn included in these

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1 Radio telemetering of electrical activity By means of this method electrical activity can be recorded in subjects which are not connected by cable to the recording apparatus and thus can move about freely

2 The introduction of electrodes dwelling chronically in brain structures

These methods can be applied in animals and exceptionally also in humans the latter method in the case of patients suffering from a brain affection necessitating the introduction of depth electrodes for diagnostic or therapeutic reasons

3 Analysis and quantification of the electrical brain phenomena and of behavioural aspects by means of computers

By means of these developments a form of liaison may be engendered between investigations of electrical phenomena of the brain and behaviour In so-doing we should realize however that the two probably do not often coincide precisely but only overlap The reason for this is that with our recording electrodes the over all average activity is derived from a number of cells and those which happen to be near to the electrodes influence them more then others which are further away The spatial representation of these cells may not be the same as the spatial distribution of the cells respon sible for the behavioural aspects The latter moreover may be influenced to a great extent by cells located at a considerable distance from the recording electrodes

The present communication is restricted to EEG radio tele metering and implantation techniques carried out in dogs with the aim of studying relationships between electrical activity in various brain structures and the animals behaviour

The electrodes were placed on various cortical and subcortical structures Of the latter the following structures have been parti cularly studied amygdalar nuclei hippocampus lateral and medial geniculate bodies medial thalamic nuclei mesencephalic reticular formation and olfactory bulb structures *Usually* 36 electrodes were introduced into the brain and connected to a plug fixed on the animals cranium In most animals it was possible to record over a period of one year or more The electrical activity was radiotransmitted by means of a small transmitter placed on the animals back and connected by cable to the plug on the head Initially 8 signals were transmitted simultaneously but recently

investigations the action of the labyrinths and the cerebellum Rademaker was particularly interested in the brain stem nuclei and the influence of cortex on these nuclei

All these investigations were carried out mainly by means of experiments in which part of the central nervous system was extirpated and the effect of the extirpations on behaviour was studied *in comparison to the behaviour before the operation*

For these investigators it was impossible to study electrical activity of the brain because the studies were hampered by practical difficulties

1 The apparatus was such that recording could not take place in freely moving animals therefore the animals had to be anaesthetized and most of the data presented up to the Second World War was obtained from anaesthetized animals or animals under other unphysiological conditions

2 In humans in the large majority of investigations the recording electrodes had to be placed on the intact scalp Only exceptionally was it possible to place recording electrodes directly in contact with the brain In humans also the recording apparatus did not allow recording during movement Though the subjects did not have to be anaesthetized they were nevertheless tied to a couch or a chair by means of the connecting wires to the apparatus Therefore in human subjects the behavioural *possibilities* were also restricted

Thus comparatively little research has been carried out into *the relationship between electrical activity and* behaviour A very large number of investigations has been carried out into electrical activities of the brain of larger as well as of smaller structures and of the brain unit the neuron In addition a large number of investigations has been carried out into the behaviour of single animals of animals in groups and of animals in colonies The specialisation has been conducted to such an extent that the electroneurophysiologist scarcely knows or understands the work of the behaviour investigators the ethologist the ecologist the psychologist and the psychiatrist

Because of the above it is advantageous *that in the last decade* methods and techniques have been developed enabling us to study electrical activities in freely moving individuals human as well as *animal* These developments concern particularly

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The present communication is restricted to EEG, radio telemetering and implantation techniques carried out in dogs with the aim of studying relationships between electrical activity in various brain structures and the animals' behaviour

The electrodes were placed on various cortical and subcortical structures Of the latter the following structures have been particularly studied amygdalar nuclei, hippocampus lateral and medial geniculate bodies medial thalamic nuclei mesencephalic reticular formation and olfactory bulb structures Usually, 36 electrodes were introduced into the brain and connected to a plug fixed on the animals' cranium In most animals it was possible to record over a period of one year or more The electrical activity was radiotransmitted by means of a small transmitter placed on the animals back and connected by cable to the plug on the head Initially 8 signals were transmitted simultaneously, but recently

an apparatus for transmission of 16 channels has been developed and put into action.

The animals' behaviour was evaluated by recording a verbal report, by taperecording televised pictures and by recording other biological phenomena (heart rate, eye movements, tail movements etc.).

In this way electrical activities in various brain structures have been observed which are clearly related to behavioural activity. These electrical activities are characteristic,

a. for their frequency or wave duration,
b. for their localisation in certain brain structures,
c. for their form and
d. for their reactivity in relation to behaviour.

For these reasons – in analogy to terminology used in clinical electroencephalography – these activities have been named "specific activities". Altogether 11 such specific activities were distinguished.

Some of these specific activities observed in dogs are also known in human electroencephalography. Others have not yet been observed in man, mainly perhaps because some of these activities occur in brain structures whose electrical activities do not appear at the scalp.

It is of importance to mention here that some of these specific activities exhibited mutual relations. These relations were of two forms, the one was called "coincidence" and the other "alternation".

Coincidence is used to indicate that some of the specific activities often occurred at the same time.

Alternation is used to indicate that some other specific activities rarely occurred at the same time: if the one was present the other was not and *vice versa*.

Apart from studying the spontaneous activities, responses to repetitive sensory stimulation were also examined. One of the observations derived from this examination was that in some cases relations existed between some of the specific activities and the responses to sensory stimulation.

In the following account a few examples will be presented of specific activities, their mutual relationships and their relationship to evoked responses.

Lambda waves, are isolated waves with a duration of approxim

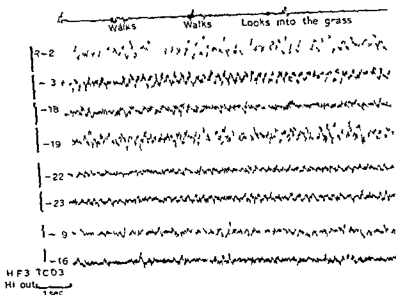


Fig 1

Simultaneous occurrence i e coincidence of theta rhythms at 5-6 c/sec in hippocampus (R-2 and R-3) and lambda waves in visual cortex (R-9) while dog was walking outside in the sun searching for something in the grass (R 18 R-19 are medial thalamic structures R-22, R 23, ventral hippocampus and R-16 olfactory bulb) Calibration 200 μV, TC = 1 sec, HF = 70 c/sec

ately 1/7-1/10 sec occurring in the visual cortex when the dog is making eye movements while in brightly lit surroundings (Fig 1) Some of the lambda waves were monophasic, others biphasic Not all eye movements were accompanied by lambda waves The most pronounced lambda waves occurred when the dog appeared to be looking with interest, as for example when walking outside in the grass or searching for something

Hippocampus theta rhythms are rhythmic waves at 5-6 c/sec occurring in various parts of the hippocampus when the dog was alert or when it was walking and particularly when it was walking to a specific goal On some occasions the frequency 5 c/sec predominated at others 6 c/sec If for example, the dog was running after a ball or a piece of meat which had been thrown away, the frequency was 6-7 c/sec when it had caught the ball or the meat and walked away the frequency dropped to 5 c/sec (Fig 2)

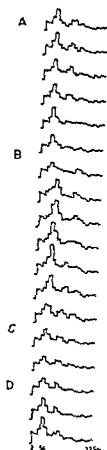


Fig 2

Series of 18 successive frequency spectra of hippocampus theta rhythm in dog Analysis period ½ sec total duration therefore 9 sec Each spectrum consists of 20 frequencies between 2 and 32 c/sec (see lowest spectrum) At A dog looked at meat, dominant frequency was 5 c/sec At B meat was thrown and dog runs after it, dominant frequency shifts to 7 and then to 6 c/sec At C dog caught meat and ate it At D dog returned dominant frequency was 5 c/sec

Hippocampus theta rhythms and lambda waves often occurred at the same time These two phenomena therefore, often *coincided* (Fig 1)

Irregular 10–20 c/sec activity in amygdala

This specific activity consists of irregular waves at frequencies between 10–20 c/sec in amygdalar structures and occurred if the dog was sniffing This activity did not occur on all occasions when the dog was sniffing but only if at the same time no lambda waves were present The irregular 10–20 c/sec activity in amygdala and the lambda waves therefore exhibited the phenomenon of "alternation" (Fig 3)

Responses evoked to rhythmic photic stimulation

In many dogs, responses evoked in various structures of the visual pathway were very variable The variability in part was

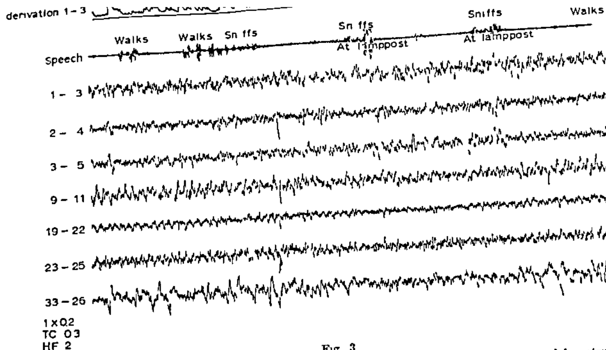


Fig 3

Alternation of lambda waves in visual cortex (vis c) and irregular 10–20 c/sec activity in amygdalar structures (amygd) while dog was sniffing Upper trace represents energy produced by amygdalar derivation 1–3 Note increase of activity in this derivation while no lambda waves are present in the visual cortex

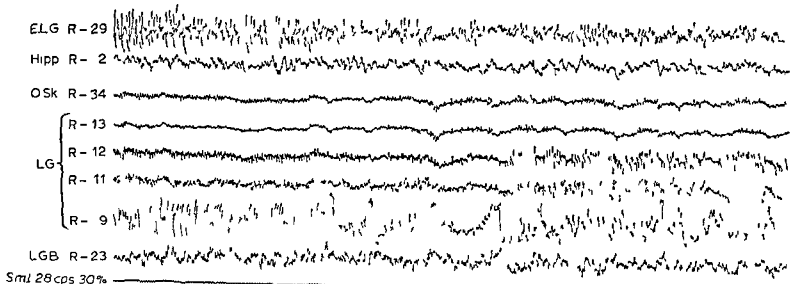


Fig 4

Alternation of lambda waves in one area of visual cortex (R–9) and responses evoked by *continuous rhythmic light stimulation* Abbreviations E L G = Ecto Lateral Gyrus, Hipp = *hippocampus*, O sk = occipital skull, L G = lateral gyrus L G B = lateral geniculate body, Stimulation sinusoidally modulated light at 28 c/sec, 30 % modulation depth The lambda waves occurred most clearly in derivation R–9 During each lambda wave the *response amplitude* decreased temporarily in derivation R–12 This is particularly noticeable in the second part of the figure when, for no known reason the response amplitudes decreased in derivation R–9 and increased in R–11 and R–12

associated with the dogs' condition but in some cases it appeared to be related to a specific activity In Fig 4 responses to continuous rhythmic light stimulation at 28 c/sec are shown in various areas of the visual cortex and in the lateral geniculate body For no apparent reason, at one moment the responses in the cortical derivation R–12 increased in amplitude while they decreased in the neighbouring cortical derivation R–9 In the latter lambda waves occurred prominently It was observed that in the period of increased response in R–12 each lambda wave in R–9 was accompanied by a short decrease of amplitude is R–12 This phenomenon was also encountered many times, in various other dogs and it was concluded that alternation occurred between lambda and visual evoked responses in some cortical areas

On the basis of these and other observations the impression was formed that the specific activities represent a state in which certain brain structures exist This state may correlate with a certain form of behaviour and may represent the condition necessary for the development of this behaviour

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MICROVASCULAR LESIONS AS A TARGET OF ANTI-INFLAMMATORY AND CERTAIN OTHER DRUGS

BY

I L BONTA

1 INTRODUCTION

1 1 SCOPE AND LIMITATIONS

This paper will deal with certain results and views which are based on our laboratory's research over the past five years, research which, it is hoped, has made some contribution to the continuing search for improved drugs for the control of inflammatory disorders

In this context the reader is reminded that the therapeutic value of some prototype anti inflammatory drugs (e g aspirin, phenylbutazone, corticosteroid) was first established by clinical empiricism rather than by well considered research on animals, even today, following many years of successful experience with such preparations in human therapy, the pharmacologists are still trying to find the best correlating experimental models, which may serve as tools for understanding the mode of action of anti inflammatory drugs and in due course may provide more efficient approaches to the synthesis and screening of new compounds for this purpose During a study of recognized anti inflammatory drugs, even in fairly simple experimental designs, the pharmacologist is still frequently faced with puzzling questions some of which, encountered during research over the past five years in this laboratory, will be cited in these pages Restricted though the scope of this paper may be when compared with the whole field of anti inflammatory drug research, no attempt can be made to give full coverage even of this aspect, the present paper is therefore little more than a composition of capita selecta A few not entirely substantiated generalizations will be unavoidable Many results to be discussed here have already been published elsewhere, but others have not Methodological details will be omitted, so as to restrict the length of the paper

1 2 Terminology

For insiders in this research field, anti inflammatory agents comprise an unambiguous class of drugs Yet if we consult a comprehensive and widely approved textbook such as Goodman and Gilman s Pharmacological Basis of Therapeutics", we shall search in vain in the Subject Index for the term anti inflammatory

This apparent contradiction deserves some consideration One could simply assert that an anti inflammatory agent is a compound which either in experimental models or in clinical use prevents or counteracts inflammation The latter however is a dynamic process rather than a stationary condition since the inflamed site undergoes continuous changes and so far there is no single drug known, which in animal experiments can antagonize all phases of the inflammatory reaction

There are quite a few drugs known, belonging to a variety of classes (depressants and stimulants of the CNS, MAO inhibitors,

hypotensive agents, smooth muscle spasmolytics) which have been reported to counteract certain types or phases of experimental inflammation in animals, though these drugs have no anti-inflammatory value in clinical use. The majority of drugs currently described as non-steroidal anti-inflammatory agents have so far scarcely been used for *controlling inflammatory disorders* other than arthritic and related conditions. By contrast, the anti-inflammatory corticosteroids are used *with increasing frequency* in inflammatory diseases in such fields as dermatology and opthalmology. *It thus appears that while the term anti-inflammatory* correlates fairly well with clinical usefulness as far as the steroidal agents are concerned, its use with respect to non-steroidal drugs is more or less limited by custom to antirheumatic agents. It is *also striking that while anti-inflammatory steroids belong to* a single, though broad chemical class possibly also having a common mode of anti-inflammatory action and differing mainly in potency or side effects, the non-steroidal anti-inflammatory drugs comprise a number of chemically unrelated compounds and it is a much disputed question as to whether they have a common mechanism of action. With these considerations in mind the omission of the term "anti-inflammatory" as a textbook Subject Index term seems to us justifiable for the time being. As far as the research field is concerned the term is as vague as is our knowledge to date on the drugs which it covers. Whatever the reason may be for using this term, for the present we have no better substitute for it.

In this paper the expression *non-steroid anti-inflammatory drug* will be applied to compounds which counteract in experimental situations one or the other component of the inflammatory reaction and which in addition have clinically recognized value as antirheumatic agents in the broadest sense of the latter term.

1.3 THE CHOICE OF MICROVASCULAR LESIONS FOR THIS STUDY

A long established difficulty in the animal study of anti-inflammatory drugs is the fact that *these agents do not in the healthy* organism exert effects which are at the present state of knowledge clearly related to their therapeutic use. Their *in vivo* pharmacological effects in healthy animals are often toxic manifestations where they have *effects other than toxic ones we so far have* no means of detecting, let alone of measuring such effects. Though

in the past few years some investigators have concluded that effects in certain biochemical systems (e g uncoupling of oxidative phosphorylation, stabilization of proteins towards heat coagulation) are well correlated with clinical anti inflammatory effects, these statements have not yet been convincingly substantiated There is thus an undeniable need for the use of appropriate animal models of inflammation Indeed, one of the crucial questions in this area of pharmacology is whether an experimentally induced inflammation in fact provides adequate simulation of the pathophysiological events underlying the clinical disorders As this need has long been recognized by pharmacologists, it explains their many efforts to produce such models, many of the existing inflammation models have been primarily developed by pharmacologists rather than by experimental pathologists For the same reason pharmacologists, though not primarily interested in exploring the mechanism of inflammation, have provided essential contributions to this end

Inflammation is a process composed of a long and even ramifying series of events For practical reasons it is important to find a reasonable middle road between selecting a model representing too narrow an aspect of inflammation and one which mimics a broad, random selection of inflammatory changes There is an acute and chronic phase of inflammation In the early phase the events have much in common, irrespective of the nature of the noxious *stimulus*, whereas a greater variance in the response may be observed in the delayed phase Redness, heat, plasma exudation, primary pain, white blood cell migration and occasionally local haemorrhage dominate the acute phase, while cell debris accumulation, fibroblast proliferation deposition of collagen fiber, tissue shrinkage, partial necrosis, secondary pain (due to organ deformity) and total repair may all occur in the chronic phase The early response is sometimes termed the vascular phase and the late response the cellular phase, though others (SPECTOR and WILLOUGHBY, 1963) discriminate even within the acute event between an exudative and a cellular phase, clearly meaning thereby that white blood cell migration represents a mechanism separate from the rest

Since however the lymphocytes are regarded as cell precursors of the fibroblasts (DUMONT, 1965), we may well consider the cellular phase of the early response as a transitional stage to the late response, the white blood cells being the connecting link All other

phenomena in the acute phase can be explained on the basis of local vascular alterations, these being partly limited to functional changes but also involving certain structural alterations It thus seems justified to say that microvascular disturbances occupy a central position in the mechanism of the acute phase in the inflammatory reaction and it was for this reason that we took these disturbances as the target for our studies

As the vessels at the inflamed site may undergo a variety of changes, it was thought appropriate to classify the studies according to the severity of the lesions Therefore the investigations presented were performed in experimental situations which more or less reflect different degrees of vascular response as follows

1 *Erythema*, the first and occasionally the only external sign of inflammation The local vessels are dilated without any gross change in structure and without major fluid leakage
2 *Oedema* being the exudative manifestation of inflammation There is a local hydrostatic imbalance and the vessel wall – be it blood or lymph vessel – may undergo such changes in structure as to allow fluid leakage
3 *Haemorrhage* which is known to accompany a variety of allergic inflammations The changes of the vessel wall together with destructive alterations in the perivascular ground substance are of such degree that not only fluid but erythrocytes leak out as well

The pharmacological aspects of the phenomena as shown above will now be discussed in that order It will be seen how our preoccupation *with investigating anti inflammatory drugs* may also contribute to a better understanding of inflammation itself The emphasis will be laid on non steroidal anti inflammatory drugs but for the sake of comparison other agents will be discussed as well

2 ERYTHEMA

2 1 LIMITATIONS OF KNOWLEDGE ON FACTORS GOVERNING ERYTHEMA

Redness of the skin as an early manifestation of inflammation and resulting from local vasodilation is readily observable in the human skin but in animals factors such as hair coverage and

colour of the skin have limited the use of this phenomenon to albino rats and guinea pigs For practical reasons we speak mostly of local vasodilation as the cause of erythema, one has however to realize that in fact the nutrient arterioles and the effluent veins may be dilated rather than the capillaries (ZWEIFACH, 1965) However uncomplicated the mechanism of erythema appears, the means to produce it in animals are few There are, naturally, plenty of agents which can induce hyperaemia, but only a few of these cause a reaction limited to erythema without accompanying increase of capillary permeability A variety of chemical materials, – such non specific irritants as croton oil or xylene, vesicants, physiological mediators including histamine or bradykinin – either when applied topically onto the skin or intradermally injected, will for example cause erythema, unfortunately, as indicated by dye extravasation techniques, plasma exudation due to permeability increase occurs simultaneously with the hyperaemia By contrast, when erythema was caused by exposing the skin to ultraviolet (UV) irradiation, a simultaneous skin blueing test was reported by originators of the method to be negative (WINDER *et al*, 1958) At a certain stage of the work in this laboratory, some doubt arose as to whether the reaction of the guinea pig skin to UV irradiation is indeed limited to vasodilation without permeability changes and hence a few experiments were performed to check the results of others We observed entirely confirming Winder's description, that the Evans blue test was negative 2 h after irradiation, but at the delayed time point of 24 h there was definitely observable dye extravasation Since at 2 h the erythema is at its maximum and accordingly this is the commonly used standard point in time for establishing drug effect in anti inflammatory testing, we may accept the statement that the UV erythema test is, for the known experimental situations the purest measure of the vasodilator event of inflammation There is only one chemical irritant known, namely thurfyl nicotinate (tetrahydrofurfuryl nicotinate), which is reported to cause a reaction in many respects similar to that induced by UV irradiation (HARVING 1963)

Except for the observations described above, astonishingly little work has been devoted to exploring the finer physiological background of the UV-erythema reaction The paucity of publications on this aspect is particularly striking when compared to the tre-

mendous efforts expended in exploring the mechanism of other inflammatory models currently used in pharmacology The fact that no change in permeability but only vasodilation occurs at the early time point after UV irradiation is an argument in favour of the assumption that in this erythema of the guinea pig no major role is played by liberation of such mediators as e g histamine plasma kinins serotonin or leucotaxin since these are known to cause increase of capillary permeability These mediators may at best be responsible for the late permeability response in ultraviolet injury Unpublished studies in this laboratory have shown that guinea pigs were not rendered unresponsive to UV irradiation when they were treated with compound 48/80 polymyxine B or reserpine i e agents known to deplete histamine and serotonin stores No attempt was however made to determine by histochemical or any other means whether the stores of the animals were indeed completely discharged The release of Slow Reacting Substance (SRS) by UV injury is a possibility as it has potent effects on smooth muscles (obviously involved in vasodilation) it does not increase vascular permeability in the guinea pig (SPECTOR and WILLOUGHBY 1962 ZWEIFACH and NAGLES 1961) though it may do so in the rat (Vargaftig unpublished) and evidence is accumulating for its being an inflammatory and/or allergic mediator

It appears that there is a real need to investigate more exactly which mediator if any is released after UV irradiation so as to cause the hyperaemic response This would not be an easy task in view of the difficulties involved in extraction and bioassay UV light is reported to cause lysosome disruption *in vitro* (WEISMANN and DINGLE 1961) but whether this occurs in the living guinea pig skin is unknown Enzymes involved in glycolytic and oxidative processes have recently been thought to play a role in the UV erythema of guinea pigs and inhibition of these enzymes was found to be correlated to antierythema action (GÓRÓG and SZPORNY 1964) The latter conclusion has been criticised (WINTER, 1966)

2 2 PHARMACOLOGICAL INHIBITION OF ERYTHEMA

UV erythema was initially used for pharmacological studies with rats but this species is not very prone to display the hyperaemic response and WILHELMI (1950) therefore introduced albino guinea pigs for the purpose The method used on guinea pigs was con

siderably improved by WINDER (1958), who made a particularly thorough study on it His method has obtained wide acceptance and is used in a practically unmodified form in this laboratory as well The original description is exceptionally good, so that no difficulty in reproducing it is encountered and experience here over a couple of years showed that even without the use of a large number of animals it is easy to obtain dose dependent results For a proper understanding of the data to be discussed it must be emphasized that no drug has ever been described as having the ability, when administered after induction of the erythema, of reversing it, and even when the most active compound is administered prior to irradiation, a fully developed erythema will inevitably show up at the 24 hour time point The inhibition due to a drug is easy to establish when the erythema is rated 2 hours following the irradiation In other words, drugs can delay, but not block or eliminate (i e "cure") the erythema

The originators of the test (WINDER *et al*, 1958) found that of more than a hundred chemical agents studied (including antihistamines, drugs acting on autonomic and central nervous system, ACTH and anti inflammatory corticosteroids) only aminopyrine, phenylbutazone, salicylates and some cincophens delayed erythema development in non toxic doses It was stated that the test can specifically predict antirheumatic activity of non-steroidal agents

We have results which indicate that the interpretation of the results obtained with this method may need revision As seen from Table 1 the four non steroidal anti inflammatory drugs (listed at the beginning of the Table) possess erythema inhibitory activity in doses which are low as compared to those required in the inflammatory oedema assay

In addition there is no major discrepancy between the potency concerning erythema inhibition and anti rheumatic value of these drugs

We do not know however by which mechanism these agents counteract the erythema response and one cannot rule out the possibility that the correlation with the clinical effect is [illegible]

[illegible] shown to produce intracranial vasoconstriction

mendous efforts expended in exploring the mechanism of other inflammatory models currently used in pharmacology The fact that no change in permeability but only vasodilation occurs at the early time point after UV irradiation is an argument in favour of the assumption that in this erythema of the guinea pig no major role is played by liberation of such mediators as e g histamine plasma kinins, serotonin or leucotaxin, since these are known to cause increase of capillary permeability These mediators may at best be responsible for the late permeability response in ultraviolet injury Unpublished studies in this laboratory have shown that guinea pigs were not rendered unresponsive to UV-irradiation when they were treated with compound 48/80, polymyxine-B or reserpine i e agents known to deplete histamine and serotonin stores No attempt was however made to determine, by histochemical or any other means whether the stores of the animals were indeed completely discharged The release of Slow Reacting Substance (SRS) by UV-injury is a possibility, as it has potent effects on smooth muscles (obviously involved in vasodilation), it does not increase vascular permeability in the guinea pig (SPECTOR and WILLOUGHBY, 1962, ZWEIFACH and NAGLES, 1961), though it may do so in the rat (Vargaftig, unpublished) and evidence is accumulating for its being an inflammatory and/or allergic mediator

It appears that there is a real need to investigate more exactly which mediator, if any, is released after UV irradiation so as to cause the hyperaemic response This would not be an easy task in view of the difficulties involved in extraction and bioassay UV light is reported to cause lysosome disruption *in vitro* (WEISMANN and DINGLE, 1961) but whether this occurs in the living guinea pig skin is unknown Enzymes involved in glycolytic and oxidative processes have recently been thought to play a role in the UV-erythema of guinea pigs and inhibition of these enzymes was found to be correlated to antierythema action (GÓRÓG and SZPORNY, 1964) The latter conclusion has been criticised (WINTER, 1966)

2 2 PHARMACOLOGICAL INHIBITION OF ERYTHEMA

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TABLE 2

Topically applied dexamethasone in guinea pig erythema and rat paw *oedema* test

Dexamethasone per cent	Erythema inhibition	Oedema inhibition
0 005	0 %	2[illegible] %
0 02	not tested	50 %
0 05	0 %	not tested

Methysergide showed the greatest antierythema activity being effective in the lowest dose of all the drugs tested here Though methysergide is a strong antagonist of serotonin it is not likely that this alone would explain its erythema inhibitory effect Firstly, because serotonin is not likely to be a mediator in the erythema response and secondly because cyproheptadine – being also a potent antiserotonin drug – was much less effective in the erythema test Methysergide has some vasoconstrictor effect and possibly a combination of this together with some as yet unrecognized property of it resulted in its marked antierythema potency Cyproheptadine has antiserotonin antihistamine and some anticholinergic activity Its vascular effects are poorly documentated but in this laboratory we found no evidence of its being a vasoconstrictor (Saxena unpublished) Which of its many effects is the main factor in its anti erythema activity is hard to say Even more complex is the situation with chlorpromazine as in addition to the effects mentioned with cyproheptadine it has a definite effect on the CNS What however needs emphasis is the fact that none of these *three* drugs has clinical anti rheumatic value and hence their activity in the erythema test strongly militates against the original statement of Winder that this method has *specific* predictive value for anti rheumatic activity of non steroidal drugs Methysergide has an outstanding reputation for use in migraine In the latter disorder a *number of inflammatory* components are by now recognized (SICUTERI 1966) and indomethacine proved in a trial to have a remarkably beneficial action on vascular headache (SICUTERI 1965)

Finally some remarks about the copper compound in Table 1 This substance along with a series of copper chelates was found in this laboratory, to have pronounced anti inflammatory activity

TABLE 1

Erythema inhibition on guinea pig
Drugs administered orally, except where otherwise shown

Compound	Erythema 50 % inhibition mg/kg	Kaolin rat paw oedema 50 % inhibition mg/kg	Ratio erythema/ oedema inhibition dose
Indomethacin	2	5 *	< 1
Phenylbutazone	7	70	< 1
Mephenamic acid	50	50 <	< 1
Aspirin	65	250	< 1
Dexamethasone	4	0.5	1 <
Hydrocortisone acetate	130	20	1 <
Methysergide	1		
Cyproheptadine	17	30	< 1
Chlorpromazine	18		
$Cu(OH)_2CuCO_3$	25	inactive at 20	< 1
$Cu(OH)_2CuCO_3$ **	inactive at 25	20 *	1 <

* Approx. ** Subcutaneous

in man (SIGUILRI 1965) and vasoconstriction is a conceivable explanation of its effectiveness in UV light induced hyperaemia.

Results with the corticosteroids likewise do not save us some speculation. Their relatively feeble antierythema potency when compared to their activity against the inflammatory oedema of the rat paw is very evident and the discrepancy is particularly striking with dexamethasone. Nevertheless the statement by Winder that non toxic doses of anti inflammatory steroids are inactive in the erythema assay appears to us an oversimplification. It was shown in a recent study that corticosteroids topically applied on human skin produce pallor due to vasoconstriction (BAKER and SATTAR 1968). We felt that dexamethasone might also produce an antierythema effect in the guinea pig when topically applied. The negative results however are clear from Table 2. We are faced with the paradoxical facts that corticosteroids cause vasoconstriction in human skin but have little effect on the erythema of guinea pigs, while the opposite is true for several anti inflammatory agents of the non steroid class. Studies on species other than the guinea pigs are therefore indicated.

inhibitory effect of drugs can be observed It is however not correct to assume that this test could specifically predict the anti rheumatic clinical value of non steroidal compounds, since some drugs devoid of anti rheumatic effect show marked erythema inhibition in the guinea pig

3 The feeble anti-erythema effect of corticosteroids on guinea pigs does not correlate with their marked pallor producing effect on human skin

3 OEDEMA

3 1 PHYSIOLOGICAL BASIS OF OEDEMA

Three main types of change will lead to oedema

a Flow increase on the arterial side *simultaneously with* constriction on the postcapillary venous side will increase the *hydrostatic* pressure in the capillaries so that fluid is extruded

b Capillary wall changes which enlarge the pores, or pericapillary basement membrane changes will allow plasma escape It was previously believed that the endothelial cell *membrane itself* underwent changes leading to escape of plasma but the present view is that the *intracellular* pores open (MAJNO and PALADE, 1961)

c Increase *in* colloid osmotic pressure outside the capillary wall or decrease of colloid osmotic pressure inside the vessel bed will also lead to fluid leakage

These changes may occur separately but also in combination with *each other* Zweifach believes that the hydrostatic mechanism may precede the capillary wall change (ZWEIFACH, 1965) We know that the changes *mentioned* under (a) and (b) *regularly* occur in inflammation Several physiological substances (e g histamine, serotonin plasma kinins Slow Reacting Substance leucotaxin, plasma globulin permeability factor etc) are known to cause one or the other of these changes and because of this some of these substances are called *inflammatory* mediators The third mechanism causing oedema is not commonly associated with inflammation, but occurs as a result of massive plasma protein loss, (e g renal disorders or during prolonged starvation) Sometimes this is not a localised oedema, but one occurring at several sites *in the body*

in the rat paw oedema test when parenterally administered. It was for this reason that the compounds were investigated in the UV-erythema method and the result with one of them, as a representative example, is shown in Table 1. In the oedema test most of the copper compounds were poorly active when administered orally and with the parenteral route their anti-inflammatory effect was regularly associated with marked tissue irritation at the injection site. It was concluded from these results that the copper compounds may not have induced the anti-inflammatory effect specifically themselves, but that this was due to non-specific tissue irritation at sites remote from the induced inflammation. Data in Table 1 clearly show that in contrast to its profile in the rat paw test, in the guinea pig UV-erythema method the copper compound was inactive subcutaneously, but active when administered orally. In the guinea pig, as in rats, it produced very marked irritation at the subcutaneous injection site. If in the oedema test the effect of this compound is indirect and due to the subcutaneous irritation, this latter factor does not seem to play a role in the inhibition of erythema. Though the gastric mucosa of the guinea pigs treated orally with the copper substance showed harsh irritation, there is no evidence as yet whether this provides a basis for an explanation of the observed anti-erythema effect. One cannot however rule out the possibility that in the guinea pig, gastric but not subcutaneous irritation evokes the remote anti-inflammatory effect.

The possibility that some organ-specific tissue component may govern the remote anti-inflammatory effect cannot be excluded. On the other hand we have no argument to rule out the possibility that in the guinea pigs the copper substance, after being absorbed from the gastrointestinal tract, may have acted by virtue of its own effect.

In closing the discussion on erythema inhibition our views may be summarized as follows:

1. When erythema is induced by UV-light and the effect is evaluated within the first 2 h following irradiation, it is a pure measure of the vasodilator phase in inflammatory reaction. Except for this, our knowledge of the mechanism of this phenomenon is scanty.
2. With the method in the guinea pig a dose-related erythema

suitable for carrying out measurements on the same animal as many times as desired, time course studies with 9 different substances were performed (BONTA, 1965, BONTA and DE VOS, 1965, DE VOS and BONTA, 1966)

1 A group of materials, including chicken egg white, serotonin, histamine compound 48/80, bradykinin and polyvinylpyrrolidone (PVP), induce oedema which attains its peak swelling within one hour of treatment and thereafter declines rapidly (*rapid and transient type*)
2 Kaolin and carrageenin will cause swelling with a peak not earlier than 4 h and with a duration of several hours (*slow and long lasting type*)
3 *Naja naja* (hooded cobra) venom produces a swelling that appears to represent a combination of both the other types, as this oedema displays a peak 1 h after the treatment and the level remains sustained for up to 4 h, after which it slowly starts to decline (*rapid and long lasting type*)
   The latter type of oedema has more recently been observed using *Bothrops Jararaca* venom as an inducer (Vargaftig, personal communication)

Except for the snake venoms – not studied for this purpose by others – the above observations are in agreement with independent investigations carried out elsewhere (WINTER 1966)

The results suggest that in the rat paw swelling at least two distinct factors play a role (one causing rapid development and the other responsible for the delayed course) and that in certain cases both factors may be present As certain of the inducing materials e g histamine, serotonin bradykinin had been earmarked in other studies as potential inflammatory and/or allergic mediators, studies were initiated at various centres to determine whether liberation of such physiological substances may have occurred when other irritants were used to produce oedema

Depletion studies in many cases made use of the paw oedema induced by chicken egg white The rat has an inborn allergy towards chicken egg white (SELYE, 1937) and animals having their serotonin and histamine stores *depleted do not* develop oedema to a subsequent plantar injection of egg white (BONACCORSI and WEST, 1963)

The foot swelling produced by polyvinylpyrrolidone (PVP) was

simultaneously Acute inflammation is practically always associated with local oedema but oedema can be present without being part of an inflammatory process Also it follows that any experimental oedema will finally reflect vessel permeability events but the nature of the oedema will determine whether or not it is suitable for anti inflammatory drug studies

3 2 THE RAT PAW OEDEMA AS A MODEL THE VARIOUS TYPES AND THE MEDIATORS INVOLVED

In laboratory animals there are several kinds of experimentally induced acute exudative conditions known all of these being more or less representative models of oedema irrespective of the mechanism Some of them however for example a peritoneal or pleural exudate suffer from the disadvantage that to measure them exactly one has to kill the animal and that one therefore cannot follow the development of the process in time The oedematous swelling of an extremity is devoid of this drawback as it can be measured without killing or even anesthetizing the animal The rat has long been known to develop oedema easily in the loose subcutaneous connective tissue of its legs after local injection of some irritant and the shape of its hind paws facilitates quantitative determination of the swelling It is probably for these reasons that the rat paw oedema is one of the most frequently used test methods in anti inflammatory drug research The number of techniques invented to measure this oedema is hardly less than the number of pharmacologists using the test Whether this is a sign that measurement of this swelling is still problematical or in contrast so easy that each pharmacologist simply uses it as an exercise in technical ingenuity is perhaps not relevant to the present discussion We are no exception in that we too have developed a device of our own for this purpose based on measurement of the thickness of the paw A statistical design for a proper evaluation of the results has been published (BONTA and DE VOS 1965) I have so far come across 18 materials which when injected into the subplantar area of the rat hind paw will cause local oedema However the physiological or biochemical mechanism of these oedemas is not uniform though some of them have features in common One way of characterizing these oedemas is to study their time course patterns As our method of measuring the rat foot swelling is particularly

that kinin like peptides may be involved in anaphylaxis is accumulating, in addition to their postulated role in a variety of non anaphylactic inflammations From the time course of the oedema induced by cobra venom and from investigations regarding its responsiveness to antagonistic drugs we tend to assume that the initial phase of this oedema is mediated through histamine or serotonin, while its delayed phase may be due to a mechanism that is possibly common to non-allergic inflammations In the oedema caused by PVP, neither anaphylactic, nor non anaphylactic inflammatory mediators appear to play a major role

The uncertainty – inherently associated with the depletion approach – as to whether the mediator stores are indeed completely discharged, means that such experiments are seldom entirely conclusive We therefore undertook studies to examine the oedema fluid of the swollen paw for the presence of some postulated inflammatory mediators (BONTA and DE VOS, 1965, 1966, 1967, BONTA *et al*, 1967) The irritant injected paw was severed at the time of maximum swelling the oedema fluid was collected by pressure and examined for the presence of mediators The bioassay principles using the Magnus method on isolated organs were followed

The first experiments have already shown that, in oedema fluids collected from serotonin, egg white, kaolin and carrageenin treated paws, large amounts of a substance were present which unmistakebly showed the characteristics of a kinin The oedema fluids examined in greater detail were those obtained after induction by *serotonin*, *kaolin* and *PVP*

The latter was interesting, as depletion studies suggested that it was not governed by amine mediators and therefore it was not counteracted by anti allergic or anti inflammatory [illegible]

[illegible] when oedema was *produced* by serotonin or kaolin but not when PVP was the inducer (Table 3)

Researchers in the inflammatory field are at present divided into two main groups as regards their views on the kinins as inflammatory or allergic mediators One group sees the kinins as playing an important role in these conditions, since these peptides fulfil the two main conditions which must be satisfied by a possible

subject to depletion studies in our laboratory Pretreatment of the rats with the serotonin and histamine depleters polymyxin B or compound 48/80 did not prevent the oedema, nor did reserpinization These studies suggest that histamine, catecholamines or serotonin do not play a major role in the PVP oedema (BONTA and DE VOS 1966)

We also investigated kaolin oedema in depleted rats Neither polymyxin B, nor compound 48/80 treatment prevented this oedema to any extent, but there was a marked inhibition in reserpinized animals This may indicate that intact catecholamine stores are needed for development of the oedema induced by kaolin (BONTA and DE VOS, 1966) Since however reserpinized rats are sedated and the latter condition itself is known to counteract the oedema in the rat, we are not certain whether peripheral catecholamine stores are indeed involved in the kaolin oedema Guanethidine however, which causes adrenergic nerve blockade was also found to inhibit this swelling The question of the role of catecholamines in inflammatory oedema probably deserves more attention than it has hitherto received Sound postulations were made to connect the catecholamines with the plasma kinin system (ROCHA E SILVA 1964, SICUTERI, 1967), the latter also being involved in kaolin oedema

The carrageenin induced oedema does not develop in rats which were depleted of bradykininogen (GARCIA LEME *et al*, 1967) This suggests a possible role of kinin in the carrageenin oedema which shows some resemblance to the swelling induced by kaolin Reserpinization of the rats however does not affect the carrageenin oedema (WINTER 1966) No depletion studies have so far been performed with oedemas produced by the snake venoms From the time course studies and investigations on depleted rats we may see some definite correlation between the various oedema types and the physiological mediators which may be involved *Serotonin* and *histamine* considered as mediators of allergic conditions appear to govern the rapidly developing swelling, except that caused by PVP These two mediators do not seem to be involved in the delayed type of oedema where in turn *catecholamines* may play a role *Bradykinin* itself causes a rapid oedema but depletion experiments – at any rate with carrageenin swelling – seem to indicate that it plays a role in the delayed oedema as well Evidence

(DI ROSA and SORRENTINO 1968) Evidence is thus accumulating to show that kinin is released in the rapid type of oedema (except PVP) and also in the delayed oedema The two stage oedemas induced by snake venoms have not yet been studied for kinin formation Serotonin may release histamine which in turn can activate processes leading to increased kinin forming activity (LEWIS 1964) Carrageenin itself is an activator of the kinin system *in vitro* (GARCIA LEME *et al* 1967 DI ROSA and SORRENTINO 1968) whilst this has not yet been shown for kaolin The latter however seems to work in some as yet unexplained way via catecholamine stores Since catecholamines are postulated as activators of pro teases leading to increased kinin formation (ROCHA E SILVA 1964) this may be one of the explanations for the elevated kinin activity in the kaolin induced oedema As to the role of catecholamines in inflammation opinions are divided since SPECTOR and WILLOUGHBY (1963) advanced the suggestion that they may be endogenous anti inflammatory substances However ROCHA E SILVA (1964) and ZWEIFACH (1961) believe that endogenous adrenaline may actually have a pro inflammatory effect Our experiments seem to support the latter view As to human conditions in which increased kinin formation has been demonstrated we would mention migraine bronchial asthma and intestinal carcinoid In one study there was no increased kinin activity shown in the synovial fluid of rheumatic patients but they were taking anti inflammatory drugs (LEWIS 1967) The latter circumstance may have influenced the results

3 3 DRUGS WHICH COUNTERACT RAT PAW OEDEMA AND THE WAY THEY ACT

We turn now to the study of prototype drugs which inhibit the oedema response of the rat paw (BONTA 1965 BONTA and DE VOS 1965) We have tested such drugs over a wide range of doses but it would be impracticable to present all these results in the present paper Instead Table 4 lists their effects in a single representative dose which caused a significant inhibition in *at least one* oedema It is very easy to see from the table that cyproheptadine having anti histamine and anti-serotonin properties *inhibited* the *rapid* type of oedemas except that caused by PVP and that it had no influence on the delayed swelling

Non-steroid anti inflammatory drugs and dexamethasone did not

TABLE 3

Kinin activity in rat paw oedema fluids

Oedema inducer	*Volume* of oedema fluid ml/rat	relative increase	*Kinin* in oedema fluid ng/ml	relative increase
Saline	0.052	–	30	–
Serotonin	0.317	6	663	22
Kaolin	0.248	4.8	760	25
PVP	0.327	6.3	54	2

mediator. Firstly their pharmacological effects resemble those which characterize the acute inflammatory or allergic response and secondly they are present in a high concentration at the diseased site. The other group of investigators however deny or at any rate strongly doubt that kinins play any significant role in the mentioned conditions. One of their main arguments is that kinins are ubiquitous substances, and their presence at the inflamed site is simply due to the fact that plasma leakage, into the extravascular space, is sufficient to activate the enzymes leading to increased kinin concentration (LEWIS, 1964). Our experiments do not support the opinions of those denying the role of the kinins as mediators. If increased kinin activity were a consequence of plasma leakage or a constantly associated phenomenon, it would have been found to be high in PVP oedema as well. The experiments, on the contrary, suggest that a high kinin concentration is associated only with oedemas representing allergic or non-allergic inflammatory conditions. The physico-chemical dynamics of the oedema produced by PVP were thoroughly studied by WINNE (1964), who arrived at the conclusion that a colloid osmotic imbalance is the main factor in this oedema. The present findings that no mediators are involved in the PVP oedema are in agreement with Winne's conclusion and imply that the foot swelling caused by PVP is a representative animal model for the oedema mechanism listed under (c) in the introduction to this section. Accordingly the PVP induced oedema is not a suitable pharmacological model for inflammatory disorders.

Increased kinin formation in the rat paw was found by others in the so called "thermic oedema" (ROCHA E SILVA, 1964) and kinin release has recently been reported in carrageenin oedema

methasone and non steroid anti inflammatory drugs were without influence on the histamine- or serotonin governed swelling and on the rapid, but non allergic oedema due to PVP The delayed foot swellings were however counteracted by both classes of anti-rheumatic drugs corticosteroids and non-steroids Practice has shown in our department and in numerous other laboratories (WINTER, 1966) that without exception all known drugs of proven effectiveness in rheumatic conditions can dose-dependently counter act the delayed types of rat foot swelling In this sense, for screening new compounds this test offers the minimum risk of overlooking a potential anti rheumatic drug The statement is of course only as valid as any other statement based on empiricism

On the other hand the correlation between the responsiveness to anti rheumatic drugs of the delayed rat paw oedemas and of rheumatoid polyarthritis is so close that one is apt to assume some similarity in the mechanism of the two conditions The kinin system itself is unlikely to be the common factor, since this system is not

...whether treating the rats with oedema inhibiting drugs has any influ ence on the elevation of kinin activity in the oedema fluids Two kinds of swellings (serotonin, kaolin) and two antagonists (cyproheptadine

...administration of cyproheptadine to the rats resulted in a dose related reduction of kinin activity in the oedema fluid when sero tonin was the subplantar irritant, but not when kaolin was the swelling inducer In the latter case the kinin concentration of the fluid was reduced in rats given phenylbutazone, which in turn failed to prevent the elevation of kinin activity in oedema produced by serotonin (Table 5) For a proper interpretation of the results the following scheme of the kinin system will be of help

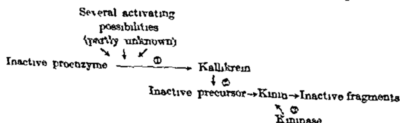


TABLE 4

Drug inhibition of different rat paw oedemas

Oedema inducer → / Dose mg/kg			Serotonin	Histamine	Cpd 48/80	Egg white	Cobra venom (early phase)	Kaolin	Carrageenin	Cobra venom (delayed phase)
omethacine	p o	5	0			0	0	++	++	+
nylbutazone	p o	100	0	0	0	0	0	++	++	+
irin	p o	250	0				0	++	++	+
amethasone	s c	0 25	0	0		0	0	++	+	+
roheptadine	p o	1	++	++	++	++	++	0	0	0
triol disodium uccinate	i v	300	+	++	+	++	+	+		+
amine P	i p	500	+	++	+	+	+	+		+

Marked inhibition (approx 50 % or more)
Moderate inhibition (25–30 % $P < 0.05$)
Insignificant inhibition or none
Empty not tested

counteract any of the rapid oedemas, but inhibited the *slowly* developing ones Oestriol disodium succinate and vitamin P (rutin) caused a non selective inhibition of all the types of swelling studied There is evidence from work in this laboratory and from others that oestriol disodium succinate decrease the vascular permeability by acting directly either on the vessel wall itself or, more likely, on the perivascular ground substance Vitamin P is also a permeability factor and its inhibitory effect on swelling produced by PVP is in agreement with the findings of others (VOGEL and MAREK 1961) Since fluid leakage through the vessel wall is the final common event in all types of oedema irrespective of the underlying mechanism the non selective inhibitor effect is understandable if the two compounds act on the vessel wall itself rather than interfering with the initiating mechanism of the swelling The fact that cyproheptadine did counteract the rapidly developing swelling corroborates the histamine or serotonin governed nature of these oedemas In turn the non responsiveness of the rapid PVP swelling and of the delayed swellings to this anti allergic drug is in harmony with the conclusion that histamine or serotonin are unlikely to be involved in these oedemas Finally it is evident that dexa

methasone and non steroid anti inflammatory drugs were without influence on the histamine- or serotonin governed swelling and on the rapid, but non allergic oedema due to PVP The delayed foot swellings were however counteracted by both classes of anti-rheumatic drugs, corticosteroids and non-steroids Practice has shown in our department and in numerous other laboratories (WINTER, 1966) that without exception all known drugs of proven effectiveness in rheumatic conditions can dose dependently counteract the delayed types of rat foot swelling In this sense, for screening new compounds this test offers the minimum risk of overlooking a potential anti rheumatic drug The statement is of course only as valid as any other statement based on empiricism

On the other hand the correlation between the responsiveness to anti rheumatic drugs of the delayed rat paw oedemas and of rheumatoid polyarthritis is so close that one is apt to assume some similarity in the mechanism of the two conditions The kinin system itself is unlikely to be the common factor, since this system is not only involved in the delayed oedema, but also in the rapid swelling, which is resistant to any anti rheumatic agent We also investigated whether treating the rats with oedema inhibiting drugs has any influence on the elevation of kinin activity in the oedema fluids Two kinds of swellings (serotonin, kaolin) and two antagonists (cyproheptadine, phenylbutazone) were studied, using the same principles for estimating the kinin activity in the oedema fluid as have been outlined

Administration of cyproheptadine to the rats resulted in a dose-related reduction of kinin activity in the oedema fluid when serotonin was the subplantar irritant, but not when kaolin was the swelling inducer In the latter case the kinin concentration of the fluid was reduced in rats given phenylbutazone, which in turn failed to prevent the elevation of kinin activity in oedema produced by serotonin (Table 5) For a proper interpretation of the results the following scheme of the kinin system will be of help

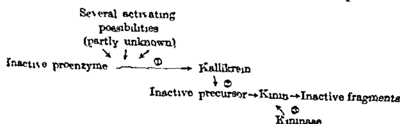


TABLE 5
Drug effects on kinin activity of rat paw oedema fluid

Oedema inducer →		*Serotonin* reduction percent		*Kaolin* reduction percent	
Drug mg/kg		*Kinin*	*Swelling*	*Kinin*	*Swelling*
Cyproheptadine s c	0 2	45	70	0	0
id	0 4	94	65	0	0
Phenylbutazone p o	50	0	10	47	33
id	200	0	0	88	80

There are three crucial phases, where changes may occur which result in high kinin concentration Two of these phases ⓑ and ⓒ lead to kinin formation and the third involves inactivation Little is known about drug effects on kininase, but the question deserves more attention than it has received hitherto As for the two activating phases in the system, the kininogen-kinin phase is known to be one step, though several enzymes from different sources (serum, urine, glands) may have kallikrein activity We cannot rule out the possibility that in cyproheptadine- or phenylbutazone treated rats the kallikrein was inhibited This however is not very likely, as neither cyproheptadine, nor phenylbutazone were found to have kallikrein-inhibiting effects *in vitro*, though for these studies the kallikrein used was of hog pancreas origin (HONDIUS BOLDINGH, personal communication)

In our view the most likely explanation for the effects of cyproheptadine and phenylbutazone should be sought at a phase even earlier than kallikrein There are several independent processes involved in this phase and one can imagine that cyproheptadine and phenylbutazone may have interfered with independent processes, both involving kallikrein activation as a final factor Whether this type of action is valid for non steroid anti inflammatory drugs other than phenylbutazone is entirely unknown For hydrocortisone however, prevention of kinin formation was reported with human leucocytes (CLINE and MELMON, 1966) and in dexamethasone treated rats the depletion of serum kininogen was also counteracted concomitantly with inhibition of the inflammatory paw oedema (GREEF, 1966)

In a number of studies it has already been shown that phenylbutazone antagonizes certain actions of kinin As a rule the broncho-

constricting effect (AARSEN, 1966), or haemodynamic effect (VARGAFTIG, 1966) evoked by bradykinin were used for this purpose All these effects however concern the antagonism of phenylbutazone towards bradykinin once it has been formed and this should not be confused with the finding that under the conditions of the inflammatory rat paw oedema, phenylbutazone treatment *in vivo* prevented the local increase of kinin formation It is certainly conceivable that as regards the clinical anti rheumatic effect of phenylbutazone either both processes occur simultaneously or that the two are coincidental and unrelated to the ultimate causal effect of this drug The same applies in the case of *cyproheptadine* but in relation to allergic clinical conditions

3 4 IS THERE A NATURAL ANTI INFLAMMATORY MECHANISM?

I wish to present here some scattered observations which seem to indicate the existence of a natural anti inflammatory process, our knowledge of which is far less than that of the drugs studied The investigations which led to these observations started with the study of a *series of metal* containing compounds, which exerted pronounced anti inflammatory effects, not only in the kaolin-induced rat paw oedema, but also for example in the cotton ball granuloma assay and the pouch granuloma test It was striking that the majority of the compounds produced marked tissue irritation at the subcutaneous injection site, and those which did not do so were also devoid of the anti inflammatory effect In addition it appeared that adrenalectomy abolished their anti inflammatory activity Some results obtained with one of the compounds are shown in Table 6 It was felt that the tissue irritation might perhaps have stressed the rats to such an extent as to activate the hypophysis adrenal axis to discharge endogenous corticosteroids, which in turn may have exerted the anti-inflammatory effect This was not very likely, as corticosterone – the main glucocorticoid produced by the rat adrenal – has a particularly feeble anti-inflammatory effect The possibility has however not yet been ruled out Nevertheless we chose to follow another train of thought, namely the assumption that local tissue irritation and remote anti-inflammatory effect may have been causally connected with each other

Support for this hypothesis was found in the literature (DIPAS-

TABLE 6

$Cu(OH)_2CuCO_3$ effect on kaolin induced rat paw oedema

Rat	$Cu(OH)_2CuCO_3$	mg/kg	Oedema inhibition percent
Intact	Subcutaneous	10	25 *
id	id	20	47 *
id	Oral	10	18
id	id	20	10
Sham oper	Subcutaneous	10	40 *
Adrenalect	id	10	12

* $P < 0.05$

Marked irritation at injection sites

In orally treated rats stomach mucosa irritated

QUALE and GIRERD, 1961, ROBINSON and ROBSON, 1966) and accordingly some experiments were started to prove or disprove this assumption

Local tissue irritation was induced by intraperitoneal injection of phenylquinone, which is known to produce abdominal writhing movements in rodents by causing acute inflammation in the peritoneal cavity When rats were intraperitoneally treated with phenylquinone 30 min prior to subplantar injection of an oedema inducer, there was marked inhibition of the swelling caused by kaolin, while the oedema induced by serotonin or PVP was not counteracted (Table 7) This seems to indicate that the remote oedema inhibitory profile of tissue irritation was similar to that of corticosteroids and non steroidal anti inflammatory drugs It was conceivable that tissue irritation may have released some factor(s) which, being discharged into the blood stream exerted at a remote site an activity resulting in suppression of the inflammatory re

TABLE 7

Interference of peritoneal irritation with rat paw oedema

Peritoneal irritation	Rats showing writhing	Hind paw oedema inducer	inhibition
Phenylquinone	8/10	Kaolin	53 %
id	9/10	Serotonin	0
id	8/10	PVP	0

sponse We therefore attempted to transfer the postulated tissue factor to other rats and examine its effect on the rat paw swelling Rats received an intraperitoneal injection of the irritant phenyl quinone, the peritoneal exudate was collected lyophylized and injected into recipient rats In another series rat paw oedema was induced by kaolin the oedema fluid was collected lyophylized and injected into other rats In both series the kaolin produced swelling served as an indicator for the anti inflammatory effect As seen from Table 8 both the peritoneal exudate and the oedema fluid

TABLE 8

Anti inflammatory effect of inflammatory exudates

Donor		Exudate * given to recipient mg/rat	Recipient		
Source of exudate	Time of collecting		Paw oedema inhibition	Adrenal weight change	Thymus weight change
Paw oedema induced by kaolin	4 h	70	22 %	0	0
	6 h	95	33 %	+ 10 %	0
Peritoneum irritated by phenylquinone	30 min	90	44 %	0	0
	1 h	145	55 %	+ 9 %	0

* Lyophilized material equivalent to 8–10 donor rats

caused oedema inhibition, these experiments seem to indicate the possible existence of a natural anti inflammatory material produced at irritated tissue sites For the moment we know little about the chemical nature of this hypothetical factor, though the lack of thymolysis and of adrenal atrophy may indicate that it is not a corticosteroid Indeed DIPASQUALE (1961) who obtained similar effects with material collected from granuloma pouch exudates, states that the anti inflammatory effect was greater when adrenal ectomized rats were used as donors We have however no experimental data which would either confirm or challenge this finding In a few subsequent experiments we obtained data to the effect that serum of irritant treated animals also exerts an anti inflammatory effect in the rat paw test, even the serum of normal animals did so though to a lesser extent

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Intact	Subcutaneous	10	25 *
id	id	20	47 *
id	Oral	10	18
id	id	20	10
Sham oper	Subcutaneous	10	46 *
Adrenalect	id	10	12

* $P < 0.05$

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id	8/10	PVP	0

since this phenomenon was discovered it still leaves us with a number of puzzling questions

Nevertheless a proper employment of this method is not only useful for rapid testing and tentative classification of new drugs for potential anti inflammatory, anti allergic and related activities, but also offers interesting possibilities for studying the mode of action of such drugs

4 HAEMORRHAGE

Permeability changes leading to haemorrhage through the vessel wall are not frequently associated with rheumatoid disorders, they are however not uncommon in a variety of allergic, anaphylactic or related inflammatory conditions It was partly for this reason, and partly because oestriol-disodium succinate – having clinical value as a vascular anti haemorrhagic drug – inhibited the rat paw oedema that we became interested in how other antagonists of the paw swelling would influence haemorrhagic vascular damage An additional motivation for these studies was that cobra venom, which induces the biphasic type of rat paw oedema, was used earlier in this laboratory to induce those particular local haemorrhages which were based on vessel wall damage rather than a coagulation defect and which were counteracted by oestriol disodium succinate (BONTA *et al* 1965, BONTA and DE VOS, 1966)

4 1 A MODEL FOR THE STUDY OF VASCULAR HAEMORRHAGE

[illegible]

would be a suitable target organ for such studies In a previous paper (BONTA *et al* 1965) the method was described in detail, but as it is not a conventional method the principles followed will, for a proper understanding of the results, be described briefly In open chest preparations of anaesthetized dogs a 5 mm filter paper disk soaked in diluted cobra venom solution is applied for 3 min to the pulmonary surface A circumscribed bleeding of non-petechial character can thus be produced on the lung due to the destructive changes evoked by the snake venom in the capillary wall The large surface of the canine lung allows one to apply the *Naja naja* venom repeatedly on one preparation. The response is dose related both with respect to onset and intensity of the haemor-

Anti-inflammatory effects were also reported with serum collected from rheumatic patients (HIGHTON, 1963) Further types of anti inflammatory factor produced by irritated tissue may well exist It would be premature at present to draw far reaching conclusions from these scattered observations, but if confirmed in more centres they may open new perspectives in anti inflammatory research They also offer a serious warning that when new compounds are tested for anti inflammatory activity, false positive results may be obtained by artifacts due to the local tissue irritant property of the compounds concerned Even oral administration does not safely exclude such artifacts, as an irritated gastric mucosa may also produce this effect, as shown by us with the copper compound in the erythema test

To conclude this discussion of rat paw oedema inhibition we have tabulated our main results with this method

Oedema inducer	Time course	Mediators involved	Antagonists	Correlated clinical condition
Serotonin Histamine Compound 48/80 Egg white Cobra venom (early phase)	Rapid (½–1 h)	Serotonin Histamine Kinin	Antiserotonin Antihistamine Direct vascular permeab in hibitors	Allergic diseases
Kaolin Carrageenin Cobra venom (delayed phase)	Slow (4–6 h)	Catechol amines (?) Kinin	Glucocortico steroids Non steroid anti inflammat ory drugs Remote tissue irritation Direct vascular permeab in hibitors	Rheumatoid diseas
Polyvinyl pyrrolidone (PVP)	Rapid (1 h)	None	Direct vascular permeab in hibitors	Non inflammatory and non allergic oedemas

We may in conclusion say that despite the simplicity of the rat paw oedema method and the long time which has elapsed

From the fact that oestriol disodium succinate counteracted one type of vascular haemorrhage but not another, we assumed that its vascular action might be more specific than originally believed We also considered that by *finding the difference in* the mode of action of the two kinds of snake venoms, this might provide us with some clue as to the actual mechanism in the effect of the anti-haemorrhagic steroid Our interest was particularly attracted towards the mechanism by which the *Naja naja* venom damaged the vessels, since at least one effect of this venom (the delayed phase of the rat paw oedema) was also counteracted by anti inflammatory drugs These *considerations led* us *to start an in*vestigation as to which of the many components and/or enzyme activities of the cobra venom accounted for its vascular damaging effect causing haemorrhage

After discussing this question, the results of studies on the ability of a variety of anti inflammatory and other drugs to antagonize cobra venom induced haemorrhages will be presented

4 2 A MECHANISM BY WHICH COBRA VENOM CAUSES VESSEL DAMAGE [1])

To clarify the haemorrhagic mechanism of the snake venoms involved we took into account those particular activities of these substances, which might be considered in first instance as factors likely to cause vascular damage On the basis of the literature (JIMENEZ PORRAS, 1968, MAY *et al*, 1966, PORGES, 1953, TU *et al*, 1967) we took the following scheme as a starting point

Property → / Venom source ↓	Hyaluronidase	Esterase	Phospholipase	Direct lytic factor (DLF)
Naja naja (hooded cobra)	+	0	+	+
Naja nigricollis (spitting cobra)	+	0	+	+
Agkistrodon piscivorus (eastern cottonmouth snake)	?	+	+	0
Crotalus adamanteus (diamond rattle snake)	?	+	+	0

+ = present, 0 = absent ? = no data

[1]) Results presented from this point onwards have a preliminary character and a full account with more details will be published elsewhere

rhage in addition to a lack of tachyphylaxis For antagonistic studies the drug is applied to the lung surface by means of the filter paper disk technique before the preparation is exposed to the snake venom Anti haemorrhagic effect can thus be established and we have indeed worked out a statistical design for calculating the significance of the inhibition obtained

Using this method we found some four years ago that oestriol disodium succinate delayed the onset and diminished the intensity of haemorrhages induced by cobra venom (BONTA and DE VOS 1966) Evidence was also presented that the observed anti haemorrhagic activity was not due to any influence on the blood coagulation system but was related to a strengthening effect on the vessel wall itself The conclusion regarding a vascular mechanism in the haemostatic action of oestriol disodium succinate was in agreement with the results of others who argued that this compound strengthens the vessel wall by promoting the formation and polymerization of the acid mucopolysaccharides in the perivascular ground substance (POLIWODA and BORNEMAN 1962) Finally the interpretation that oestriol disodium succinate induces a higher resistance of the vessel wall against damage was also in conformity with the finding that this drug inhibited all kinds of rat paw oedemas irrespective of the underlying mechanism It was expected that the compound would also exert a vascular haemostatic effect irrespective of the agent used to cause capillary wall destruction resulting in haemorrhage Nevertheless it was found (Cerquiglini personal communication) that in the hamster cheek pouch oestriol disodium succinate definitely counteracted the bleedings caused by cobra venom but failed to inhibit haemorrhages induced by the venom of *Agkistrodon piscivorus* another poisonous snake This unexpected result led us to reinvestigate it using our canine lung surface method During this investigation we corroborated the ineffectiveness of oestriol disodium succinate towards bleeding caused by *Agkistrodon piscivorus* venom In addition we also observed that the latter venom induced a haemorrhage which not only differed in its drug responsiveness but also in its macroscopic appearance from that caused by *Naja naja* venom While cobra venom produces diffuse bleeding the *Agkistrodon piscivorus* venom causes a haemorrhage having the appearance of a petechial bleeding

TABLE 9

Haemorrhagic effect of snake venoms on lung surface

Venom 2 mg/ml	Incubation	Haemorrhage
Naja naja	—	Marked, diffuse
id	heparin	None
Naja nigricollis	—	Marked diffuse
id	heparin	None
Agkistrodon piscivorus	—	Marked, petechial
id	heparin	Marked petechial

It is clear that heparin treatment (=precipitation of a basic peptide) of either *Naja naja* or *Naja nigricollis* venom entirely abolished their haemorrhagic action By contrast *Agkistrodon piscivorus* venom retained its haemorrhagic effect after incubation with heparin A number of attempts to restore the haemorrhagic action of the heparin treated cobra venoms by re adding the precipitate to the supernatant have so far yielded inconclusive results Though we cannot rule out the possibility that the DLF itself *in the cobra venoms is responsible for* the vascular action, a more likely explanation is that the presence of DLF renders the phospholipase or hyaluronidase of the venoms capable of damaging the vessel wall With the haemorrhagic action of the Agkistrodon venom this system apparently does not play a role and its phospholipase alone is not able to attack the vessels It is also unlikely that the vascular destructive effect of this venom can be explained on the basis of its esterase activity, since trypsin and kallikrein do not seem to cause haemorrhage on the lung surface Data in a recent review indicate that the haemorrhagic factor in some venoms is a protein devoid of proteolytic activity but *Agkistrodon piscivorus* venom was not mentioned (JIMÉNEZ PORRAS 1968) As the haemorrhagic action of the *Agkistrodon* venom is not dependent on a factor which can be *precipitated by* an acid polymer, the formation of mucopolysaccharides by oestriol disodium succinate is not an effective defence mechanism for protecting the vessels from the destructive action of this venom

The properties of the venoms as presented above might account for vascular haemorrhage by the following mechanisms

1 *Hyaluronidase* by depolymerization of hyaluronic acid in the surrounding ground substance of the vessels
2 *Esterase* by releasing kinins, known to damage the vessels
3 *Phospholipase* by releasing Slow Reacting Substance (SRS) or histamine through lysolecithin or both As to SRS, opinions are as yet divided as to whether it can increase capillary permeability
4. *Direct Lytic Factor* (DLF) is a basic polypeptide, discovered recently (JIMINEZ-PORRAS, 1968), it can be precipitated by heparin and has no enzymatic activity However, when added to purified phospholipase – which itself is unable to attack the intact cell membrane – it renders the enzyme capable of causing cell lysis For example, cobra venom can cause haemolysis but heparin treated cobra venom can no longer do so since the DLF in it is precipitated

In a first attempt to prove or exclude the ability of some possible factors to cause bleeding a number of substances were tested for this activity on canine pulmonary preparations The sensitity of the preparations was in each case controlled by concomitantly applied cobra venom or *Agkistrodon piscivorus* venom and without exception all pulmonary preparations were very sensitive to the haemorrhagic action of the two venoms *The following materials were however inactive in causing haemorrhage when applied to the lung surface* hyaluronidase (testicular source) trypsin, kallikrein (hog pancreas source), kinin (released from rat serum by added kallikrein), *Crotalus adamenteus* venom, compound 48/80, polymyxin-B, histamine phospholipase-A (prepared from *Crotalus terrificus* venom), purified phospholipase (from *Naja nigricollis* venom) Slow Reacting Substance (released from egg yolk by added *Naja nigricollis* venom) It appeared that these negative results ruled out any role of hyaluronidase, esterase and phospholipase as such in the action of the haemorrhagic snake venoms tested It remained necessary to test the possible role of the Direct Lytic Factor To this end both cobra venom solutions and solutions of *Agkistrodon* venom were incubated with heparin and centrifuged The supernatant fluids were tested on the lung surface The results obtained from three dogs are summarized in Table 9

However since evidence is accumulating for the existence of two kinds of histamine pool (MAY *et al* 1966), it is conceivable that the lung tissue contains only the stable pool, this being resistant to compound 48/80, but accessible to the cobra venom It appears from the drug antagonism profile (non steroid and steroid anti inflammatory drugs inactive, anti histamine active) that at least one of the mechanisms involved in the haemorrhagic phenomenon on the lung surface induced by cobra venom may be similar to the rapid phase of the rat paw oedema

At the time of completing this manuscript the studies of drug antagonism to the haemorrhagic action of *Agkistrodon piscivorus* venom have just been started, they will be the subject of another paper

5 CLOSING REMARKS

Anti inflammatory drugs can counteract certain microcirculatory lesions, while leaving others unaffected Unfortunately we know less about the mechanism of the lesions which they inhibit, than about the lesions which they do not counteract

It appears that the main difficulty in today's anti inflammatory drug research lies in the fact that, although insight has been obtained into a number of the processes involved, we do not know which if any of them is the final event, thus it is difficult to make a choice as to which process should be the target Until this is known two ways are open to us

a The screening of new compounds prepared either at random or on the basis of some theory As present knowledge on structure activity relationships with anti inflammatory drugs is possibly less than in any other major branch of pharmacology the chance of obtaining a useful anti inflammatory drug from a random chemical series nearly equals that of finding one among compounds based on a sophisticated theoretical programme

b Making a choice of one of the processes or enzyme systems supposed to be of significance for inflammation and developing a specific inhibitor of this system A high element of chance is also present in such a system-directed approach, simply because of the still existing uncertainty as to which system should be our target

4.3. Drugs which inhibit the haemorrhage induced by cobra venom

Presented below are the results of those experiments in which anti-inflammatory and certain other drugs were tested for their ability to inhibit cobra venom-induced haemorrhages.

As can be seen from Table 10, neither of the non-steroidal anti-inflammatory compounds studied showed any effect, and similar inactivity was found with *two glucocorticoids*. Apparently the mechanism involved in the haemorrhagic vascular damage on the lung surface is different from that in the delayed phase of rat paw oedema caused by cobra venom. The anti-haemorrhagic effect of topically applied heparin is in agreement with its capacity to abolish the haemorrhagic effect of cobra venom when incubated *in vitro*. It is possible, but by no means certain, that heparin may have precipitated the DLF of cobra venom on the lung surface as it did in the test tube The haemorrhage inhibiting action of the two anti-histamines was unexpected, as neither compound 48/80 nor polymyxin-B was able to produce bleeding of the lung surface, apparently ruling out a possible role of histamine release

Table 10

Drug effect on *Naja naja* induced haemorrhage

All drugs topically applied before exposing lung surface to snake venom

For each drug 8–10 observations

Drug 10 mg/ml	Haemorrhage
Oestriol disodium succ .	0
Phenylbutazone	+
Aminopyrine	+
Hydroxychloroquine sulphate	+
Calcium gluconate	+
Dexamethasone phosphate *	+
Hydrocortisone succinate	+
Desoxycorticosterone glucoside	+
Heparine	0
Phenergan .	0
Mepyramine maleate	0

* 5 mg/ml

\+ Haemorrhage marked

0 Haemorrhage strongly delayed and less intensive

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Experimental research on inflammation will however be equally necessary for both approaches, as only improved knowledge will help to find an optimal pharmacological model for screening purposes and the unravelling of the ultimate process of inflammation will provide vital guidelines in determining the system with which one may wish to interfere

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Experimental research on inflammation will however be equally necessary for both approaches, as only improved knowledge will help to find an optimal pharmacological model for screening purposes and the unravelling of the ultimate process of inflammation will provide vital guidelines in determining the system with which one may wish to interfere

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EFFECTS OF BRADYKININ AND ANTI-INFLAMMATORY AGENTS ON THE RESPONSE OF ISOLATED GUINEA-PIG LUNGS TO HISTAMINE

BY

P N AARSEN

1 INTRODUCTION

In previous experiments on vascular perfusion of isolated guinea-pig lungs it was found that the preparation soon became oedematous if bradykinin and histamine were administered alternately (AARSEN, 1966) Since both substances increase vascular permeability, oedema formation is to be expected In these experiments no inhibitory influence of non steroid anti inflammatory agents could be demonstrated either on the vasoconstrictor or on the bronchoconstrictor responses to histamine and bradykinin These findings seemed to contradict many other investigations GREEFF and MOOG (1964) found that phenylbutazone and acetylsalicylic acid inhibited the vasoconstrictor effect of both bradykinin and histamine in isolated guinea pig lungs, whereas the bronchoconstrictor effect of bradykinin was only decreased STARR and WEST (1966) demonstrated that slow infusion of various non-steroid anti inflammatory agents abolished the constrictor responses of the isolated rabbit pulmonary artery to histamine as well as to bradykinin and 5 hydroxytryptamine HAUGE, LUNDE and WAALER (1966) using isolated blood-perfused lung preparations of the dog found that high doses of phenyl[illegible] [illegible] bradykinin did not cause a significant bronchoconstrictor effect

An important cause of this discrepancy may be the condition of the lung vessels which may be determined by the composition of the perfusion fluid and by the perfusion pressure

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was connected to a Marey tambour recording the variations in amplitude of tracheal pressure As is shown in Fig 1 the cannula in the pulmonary artery was attached to a tube with three arms one arm was connected to the perfusion system, the second arm served as an outlet for air and through the third arm a thin polythene cannula was introduced to inject drugs The perfusion fluid leaves the preparation via the pulmonary veins The finger pump (3 in Fig 1) was adjusted to give a perfusion rate of about 23 ml per min In this set up the changes in perfusion pressure were recorded instead of changes in outflow The pressure in the perspex

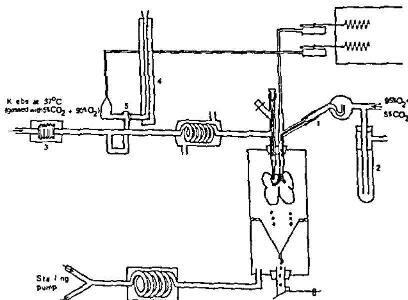


Fig 1

Diagram of the apparatus used for recording the tracheal pressure amplitude and the pulmonary arterial pressure, modified from BHATTACHARYA and DELAUNOIS (1955) The tracheal cannula is attached to a tube with a side arm through which a polythene cannula with a bore of 1 0 mm is pushed At (1) the system is filled with 95 % oxygen and 5 % carbon dioxide at a pressure of 5 cm water maintained by means of the overflow at (2) The cannula in the pulmonary artery is attached to a tube with three arms, one of these arms is connected to the finger pump (3) The water manometer (4) is used for calibration of the Marey tambour recording the perfusion pressure in the pulmonary artery by opening the stopcock (5)

In previous experiments (AARSEN, 1966) the perfusion fluid was Krebs-Ringer solution to which 3 % dextran was added It was impossible to keep the perfusion pressure constant during the experiment since the pressure had to be increased temporarily in order to overcome a persisting high vascular resistance after the addition of drugs

In the experiments to be discussed in the present paper, the vessels of isolated guinea pig lungs were perfused with Krebs Ringer solution without dextran at a constant rate of 23 ml per min by means of a finger pump Thus it was possible to avoid the difficulty met with in the former technique, in which the administration of vasoconstrictor drugs sometimes led to an almost complete occlusion of the vascular bed as a consequence of which the flow became very low and the preparation was exposed for a long period of time to the effect of high doses of the drugs Using the new technique the drugs are rapidly washed out even if they cause a considerable degree of vasoconstriction In this new set up the responses to histamine, 5 hydroxytryptamine and bradykinin added separately were first investigated Thereafter the effects of bradykinin and anti inflammatory drugs on the responses elicited by histamine were investigated

2 MATERIALS AND METHODS

2 1 ISOLATED GUINEA PIG LUNGS WITH VASCULAR PERFUSION

A modification of the method described by BHATTACHARYA and DELAUNOIS (1955) was used Female guinea pigs of 250 to 450 g were anaesthetized with 50 mg of pentobarbitone per 1 g body weight intraperitoneally After intravenous administration of 500 I U heparin both the trachea and the pulmonary artery were cannulated After removal of the lungs together with the trachea the vessels were freed from blood by perfusion with Krebs Ringer solution under low pressure The lungs were then suspended immediately in the perspex cylinder by means of the cannulae (see Fig 1) The tracheal cannula was attached to a tube with a side arm through which a polythene cannula with a bore of 1 0 mm was pushed At 1 (Fig 1) the system was filled with 95 % oxygen and 5 % carbon dioxide at a pressure of 5 cm water which was maintained by means of an overflow (2 in Fig 1) The main tube

was connected to a Marey tambour recording the variations in amplitude of tracheal pressure. As is shown in Fig. 1 the cannula in the pulmonary artery was attached to a tube with three arms: one arm was connected to the perfusion system, the second arm served as an outlet for air and through the third arm a thin polythene cannula was introduced to inject drugs. The perfusion fluid leaves the preparation via the pulmonary veins. The finger pump (3 in Fig. 1) was adjusted to give a perfusion rate of about 23 ml per min. In this set-up the changes in perfusion pressure were recorded instead of changes in outflow. The pressure in the perspex

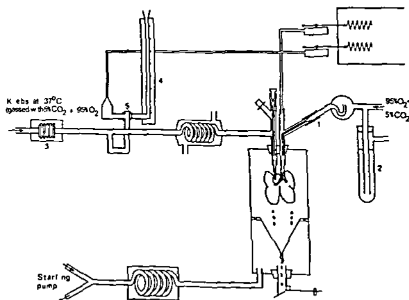


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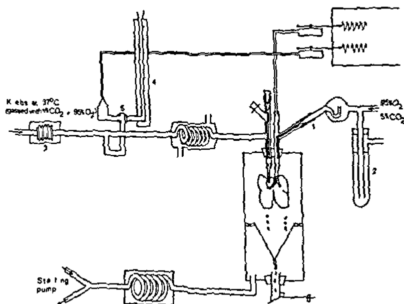


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cylinder with a capacity of about 1420 ml was alternately made positive and negative by means of a Starling respiration pump which was adjusted to give 13 strokes per min of 100 ml The e changes in pressure caused fluctuations in the perfusion pressure in the pulmonary artery between about +8 cm and −8 cm H O in most experiments

2 2 Materials

The following substances were used synthetic bradykinin (Sandoz BRS 640) histamine acid phosphate 5 hydroxytryptamine creatinine sulphate The following analgesic antipyretic drugs with anti inflammatory activity were tested sodium salicylate phenazone amidopyrine sodium phenylbutazone phenacetin and estriol succinate [1]) Only the doses of histamine and of 5 hydroxytryptamine are expressed in terms of the base All doses of histamine 5 hydroxytryptamine and of bradykinin were administered in 0 2 ml Krebs Ringer solution in 30 sec When the combined effect of bradykinin and histamine was investigated histamine was added immediately after bradykinin Immediately afterwards the cannula was flushed with 0 1 ml Krebs Ringer solution In all experiments the smooth muscle stimulating drugs or combinations of these drugs were injected into the cannula in the pulmonary artery at intervals of 10 min The anti inflammatory agents were added to the perfusion fluid and the preparation was perfused with the anti inflammatory agent for at least 20 min before the injections with the stimulating drugs were started again The anti inflammatory agents were tested at concentrations of 20 μg per ml except for sodium salicylate which was given in concentration of 40 μg per ml These concentrations correspond approximately to the therapeutic blood levels

3 RESULTS

3 1 The effect of histamine bradykinin and 5 hydroxytryptamine

In seven isolated guinea pig lung preparations the effects of two doses of histamine (x and $2x$) on the tracheal pressure amplitude and on the pulmonary arterial pressure were investigated after

1) Estriol succinate was kindly supplied by Organon Oss

repeated administration The doses varied according to the sensitivity at the beginning of each experiment in four of these experiments x was 1 μg of histamine in the others 0 5 μg A typical experiment is shown in Fig 2 The upper part of this Figure shows the effects of 1 and 2 μg of histamine on the amplitude of the tracheal pressure and on the pulmonary arterial pressure in the first and second dose cycle in the lower part the effects during the fourth dose cycle are shown In this experiment the effect of

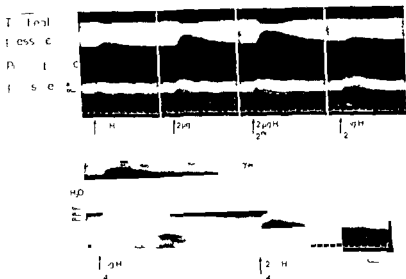


Fig 2
Isolated guinea pig lung preparation The effect of 1 and 2 μg histamine (H) on the tracheal pressure amplitude and on the pulmonary arterial pressure in the 1st and 2nd dose cycle (upper part) and in the 4th dose cycle (lower part)

histamine on both the amplitude of the tracheal pressure and on the pulmonary arterial pressure increased after repeated administration this was true both for the magnitude of the effect and for its duration It must be noted that in this set up the perfusion rate of the lung vessels is almost unaffected by an increase in vascular resistance so that an increase in the duration of an effect cannot be ascribed to a slower rate of removal of histamine Furthermore in the third and fourth dose cycle the amplitude of

the tracheal pressure did not return to its original height within 10 min Fig 3 shows a graphical presentation of the mean effects (± SEM) of four experiments in which 1 and 2 μg of histamine were administered in four cycles An analysis of variance of the results is presented in Table 1 This Table shows that the effect of histamine on the amplitude of the tracheal pressure is dependent on the dose, moreover there is a significant increase in the effect

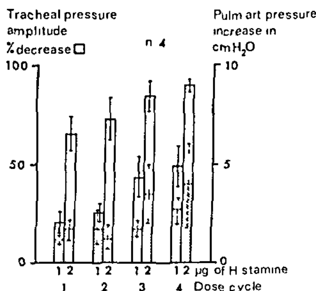


Fig 3
Mean responses of the isolated guinea pig lungs to 1 and 2 μg of histamine in four successive cycles The bars indicate ± 1 SEM

TABLE 1
Analysis of variance of the data plotted in Fig 3 showing the effects of histamine amplitude of tracheal pressure and on the pulmonary arterial pressure

Source of variation	Degrees of freedom	Tracheal pressure Mean square	F	P	Pulmonary arte[rial] pressure Mean square	F	
Doses	1	15 225 12	71 35	<0 005	13 78	2 72	>
Cycles	3	1 237 00	5 79	<0 005	13 03	2 57	>
Parallelism	3	20 08	10 65	>0 025–<0 05	7 03	1 38	>
Experiments	3	566 58	2 65	>0 05	29 78	5 88	<
Error	21	213 36			[illegible]06		

of histamine during the different cycles (in both cases $P < 0.005$) However, on the pulmonary arterial pressure no significant change in effect was found either by increasing the dose or during the different dose cycles (in both cases $P > 0.05$) This may be caused partly by the wide variation in the effect on the pulmonary arterial pressure within the different experiments ($P < 0.005$)

In a similar way the effects of 2 and 4 μg bradykinin on the tracheal pressure and on the pulmonary arterial pressure were investigated in five experiments The mean effects (± SEM) of these experiments are plotted in Fig 4

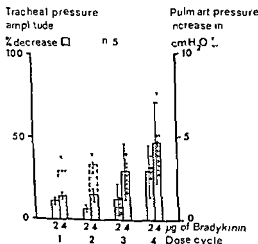


Fig 4
Mean responses of isolated guinea pig lungs to 2 and 4 μg of bradykinin in four successive cycles The bars indicate ± 1 SEM

Fig 4 shows that bradykinin has a marked effect on the pulmonary arterial pressure at doses which have a relatively small effect on the tracheal pressure Fig 3 shows that histamine had a much more pronounced effect on the tracheal pressure changes These findings are in accordance with those of HAUGE LUNDE and WAALER (1966)

Regarding the effects of bradykinin on the pulmonary arterial pressure a significant difference between the two doses ($P < 0.005$) and between the cycles ($P < 0.01$) was found

The effect of bradykinin on the amplitude of the tracheal pressure increased significantly ($P<0.05$) with successive administrations. However, this effect appeared to be independent of the dose ($P>0.05$). After repeated administration of bradykinin the amplitude of the tracheal pressure did not return to its normal height. This effect was even more pronounced with bradykinin than with histamine.

These experiments show that some of the effects of both histamine and bradykinin increase with the number of successive administrations, while eventually irreversible changes in the preparation seem to take place. Since these phenomena may be ascribed to a progressive increase in vascular permeability, it was of interest to study the effect of 5-hydroxytryptamine. Although this latter compound increases vascular permeability in the rat it was shown by WILHELM (1962) to be relatively ineffective in the guinea pig.

In five experiments three cycles of 2 and 4 μg of 5-hydroxytryptamine were investigated. The mean effects ($\pm$ S.E.M.) of these experiments are plotted in Fig. 5. This figure shows that after repeated administration the effect of 5-hydroxytryptamine on the tracheal pressure decreased rather than increased, whereas the effect on the pulmonary arterial pressure showed little change.

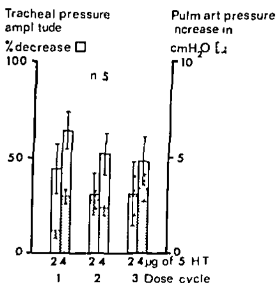


Fig. 5

Mean responses of isolated guinea pig lungs to 2 and 4 μg of 5-hydroxytryptamine in three successive cycles. The bars indicate $\pm$ 1 S.E.M.

This is in sharp contrast to the results obtained with histamine and bradykinin In a number of experiments three different doses of 5 hydroxytryptamine were given at random each dose being given three times Fig 6 shows a representative experiment The upper part of this figure shows the effects of 2, 4 and 8 μg of 5 hydroxytryptamine on the tracheal and on the pulmonary arterial pressure in the first dose cycle, the responses obtained in the third dose cycle are shown in the lower part A comparison of the effects observed during the first dose cycle with those of the third cycle shows that the effect of 5 hydroxytryptamine on

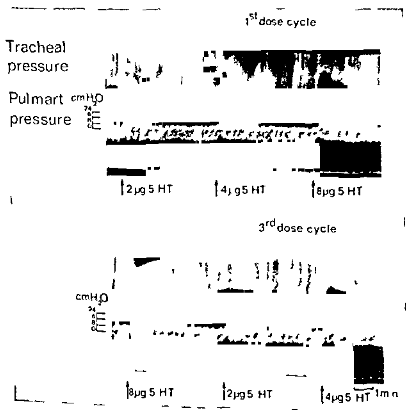


Fig 6
Isolated guinea pig lung preparation Effects of 2 4 and 8 μg of 5 hydroxy tryptamine on the tracheal pressure amplitude and on the pulmonary arterial pressure in the 1st (upper part) and in the 3rd dose cycle (lower part)

the tracheal pressure was not increased and that in the control periods between the administrations of 5-hydroxytryptamine the amplitude of the *tracheal pressure remained unaffected*.

3.2. THE EFFECT OF A SUBLIMINAL DOSE OF BRADYKININ ON THE RESPONSES TO HISTAMINE

In previous experiments (AARSEN, 1966) isolated guinea-pig lung preparations very soon became oedematous if bradykinin and histamine were administered alternately. Therefore the combination of bradykinin and histamine was tested using the new method of perfusion. Preliminary experiments showed that combinations of histamine and bradykinin in doses which were active by themselves could not be investigated: after one or two administrations the airways became completely blocked by fluid. Therefore combinations of a small effective dose of histamine with various subliminal doses of bradykinin were investigated. A dose of 50 ng bradykinin, which was about a twentieth of the liminal dose effective on the tracheal and pulmonary arterial pressure still enhanced the responses to histamine given immediately afterwards. This combination did not cause leakage of fluid into the airways even after repeated administration. In 15 experiments 0.5 and 1.0 μg of histamine were added alone in a first dose cycle followed by a second dose cycle in which half these amounts were added preceded by a dose of 50 ng of bradykinin. Since it was found in previous experiments that the effect of histamine on the tracheal pressure increased significantly after repeated administration, 0.5 and 1.0 μg of histamine were added in two cycles without bradykinin in 15 experiments serving as controls. The mean effects (± SEM) of these two groups of experiments are summarized in Table 2. This table shows that in the first dose cycle the effects of histamine on both the tracheal and pulmonary arterial pressure were about equal for the control group and for the test group.

After the subliminal dose of bradykinin was added the effect of histamine was increased in both cases. A dose of 0.25 μg of histamine, which in itself was inactive, showed an effect in combination with bradykinin. The responses to 0.5 μg of histamine in combination with bradykinin must be compared with the responses to 0.5 μg of histamine given alone in the second dose cycle of the control group. Table 2 shows that the mean responses to 0.5 μg of histamine

TABLE 2

Influence of a subliminal dose of bradykinin on the responses of isolated guinea pig lungs to histamine

First dose cycle: 0.5 and 1.0 μg of histamine was added to both the control and the test groups without previous administration of 50 ng of bradykinin. Second dose cycle: the control group received the same doses of histamine without administration of 50 ng of bradykinin. In the test group 0.25 and 0.5 μg of histamine were added immediately after the subliminal dose of 50 ng of bradykinin. Averages (± S.E.M.) of 15 experiments are presented. 95% fiducial limits in brackets.

	1st Dose cycle			2nd Dose cycle			
	Bradykinin added (ng)	Dose of histamine (μg) 0.5	1.0	Bradykinin added (ng)	Dose of histamine (μg) 0.25	0.5	1.0
Decrease in tracheal pressure amplitude in %							
control group	—	19.4 ± 3.2 (12.5 — 26.3)	56.2 ± 6.5 (42.2 — 70.2)	—		32.5 ± 5.1 (21.6 — 43.4)	69.6 ± 7.2 (54.2 — 85.0)
test group		17.6 ± 4.7 (7.6 — 27.6)	58.8 ± 6.3 (45.3 — 72.3)	50	27.6 ± 6.2 (14.4 — 40.8)	57.8 ± 6.6 (43.7 — 71.9)	
Increase in pulmonary arterial pressure in cm H_2O							
control group	—	0.9 ± 0.2 (0.3 — 1.5)	1.3 ± 0.2 (0.7 — 1.9)	—		1.7 ± 0.3 (1.1 — 2.3)	2.3 ± 0.4 (1.3 — 3.3)
test group	—	1.4 ± 0.4 (0.6 — 2.2)	1.2 ± 0.5 (1.2 — 3.2)	50	2.7 ± 0.3 (2.2 — 3.2)	3.0 ± 0.5 (2.0 — 4.0)	

in combination with bradykinin were almost twice those to histamine given alone. The analysis of variance of the results showed significant differences ($P < 0.005$).

3.3 Influence of anti-inflammatory agents on the responses of isolated guinea-pig lungs to histamine

The following antipyretic analgesics with anti-inflammatory activity were investigated: sodium salicylate, amidopyrine, phenylbutazone and phenazone. In all experiments two doses of histamine (0.5 and 1 μg) were injected into the lung vessels twice before and twice 20 min after the perfusion fluid was changed from normal *Krebs-Ringer solution to Krebs-Ringer solution containing* the anti-inflammatory agent under test. The concentrations of the anti-inflammatory agents were 20 μg per ml except for sodium salicylate which was given in a concentration of 40 μg per ml. The doses of histamine were given at intervals of 10 min. As was shown in Fig. 3 the effect of histamine tends to increase with each dose cycle. Fig. 7 shows the strong inhibitory effect of phenylbutazone and amidopyrine on the decrease of the tracheal pressure amplitude induced by histamine. Both compounds were about equally active. Phenazone was possibly somewhat less effective while sodium salicylate had a markedly smaller effect. This sequence of drug activity roughly corresponds to their potency in suppressing skin erythema caused by ultra-violet irradiation in guinea-pigs (WINDLER *et al.* 1958). The anti-inflammatory agents investigated seemed to have no effect on the increase in pulmonary arterial pressure induced by histamine (see Fig. 7). The difference between the responses to histamine before and after the perfusion fluid was changed was not significant at the 5 % level.

3.4 Influence of anti-inflammatory agents on the responses of the isolated guinea-pig lungs to histamine in combination with bradykinin

Since it was found that the sensitivity to histamine as well as the histamine sensitizing activity of bradykinin varied considerably in different lung preparations (see SLM's in Table 2), in each experiment two doses of histamine alone (first dose cycle) were given followed by the addition of half these doses in combination with a subliminal dose of 50 ng of bradykinin (second dose

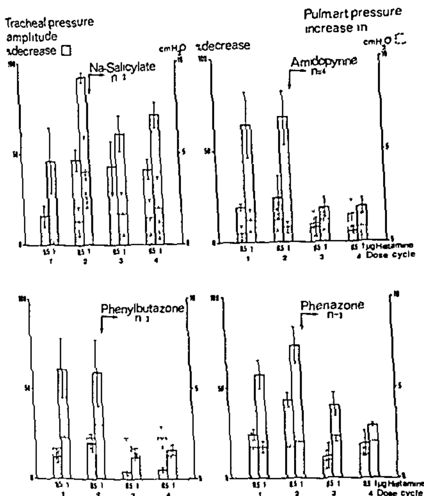


Fig 7

Influence of anti inflammatory agents on the responses of the isolated guinea pig lungs to histamine Two doses of histamine (0 5 and 1 µg) were administered twice before and twice after starting perfusion (indicated by arrow) with Krebs Ringer solution containing the drug under test Amidopyrine and phenylbutazone (20 µg/ml) predominantly inhibited the effects of histamine on the tracheal pressure amplitude In this respect phenazone (20 µg/ml) and next sodium salicylate (40 µg/ml) were less effective The effect of histamine on the pulmonary arterial pressure was not significantly suppressed by these drugs

in combination with bradykinin were almost twice those to histamine given alone. The analysis of variance of the results showed significant differences (P 0.005).

3.3 Influence of anti-inflammatory agents on the responses of isolated guinea-pig lungs to histamine

The following antipyretic analgesics with anti-inflammatory activity were investigated: sodium salicylate, amidopyrine, phenylbutazone and phenazone. In all experiments two doses of histamine (0.5 and 1 μg) were injected into the lung vessels twice before and twice 20 min after the perfusion fluid was changed from normal Krebs-Ringer solution to Krebs-Ringer solution containing the anti-inflammatory agent under test. The concentrations of the anti-inflammatory agents were 20 μg per ml except for sodium salicylate which was given in a concentration of 40 μg per ml. The doses of histamine were given at intervals of 10 min. As was shown in Fig. 3 the effect of histamine tends to increase with each dose cycle. Fig. 7 shows the strong inhibitory effect of phenylbutazone and amidopyrine on the decrease of the tracheal pressure amplitude induced by histamine. Both compounds were about equally active. Phenazone was possibly somewhat less effective while sodium salicylate had a markedly smaller effect. This sequence of drug activity roughly corresponds to their potency in suppressing skin erythema caused by ultra-violet irradiation in guinea pigs (Windler *et al.* 1958). The anti-inflammatory agents investigated seemed to have no effect on the increase in pulmonary arterial pressure induced by histamine (see Fig. 7). The difference between the responses to histamine before and after the perfusion fluid was changed was not significant at the 5 % level.

3.4 Influence of anti-inflammatory agents on the responses of the isolated guinea-pig lungs to histamine in combination with bradykinin

Since it was found that the sensitivity to histamine as well as the histamine-sensitizing activity of bradykinin varied considerably in different lung preparations (see S.E.M.s in Table 2) in each experiment two doses of histamine alone (first dose cycle) were given, followed by the addition of half these doses in combination with a subliminal dose of 50 ng of bradykinin (second dose

cycle) After these preliminary tests the lungs were perfused with Krebs Ringer solution with or without the addition of the drug under test Twenty minutes later the response to the combination of 50 ng bradykinin with the highest dose of histamine was tested again (third dose cycle)

Fig 8 shows a typical experiment In the control experiment (upper section) the effect of 0 5 μg histamine in combination with bradykinin on the tracheal pressure is strongly increased in the third dose cycle This response was almost completely suppressed by amidopyrine (middle section) and phenylbutazone (lower section) in concentrations of 20 μg per ml perfusion fluid Phenazone was found to be less effective while sodium salicylate produced a slight effect if any

Phenylbutazone was also compared with phenacetin and estriol both in a concentration of 20 μg per ml Phenacetin was chosen as

TABLE 3

ison of the effect of phenylbutazone with those of phenacetin and estriol on the response of isolated guinea pig lungs to histamine in combination with bradykinin

se cycle 0 5 and 1 0 μg of histamine were added alone

again 20 min after the perfusion with 20 μg/ml of the drug under tes was started

ean responses to 0 5 μg of histamine with and without bradykinin are compared Th responses to the combined administrations in the second and the third dose cycle ar expressed as multiples of those to histamine alone in the first cycle

er Drug	Mean responses ($\pm$ SEM) to 0 5 μg histamine (1st dose cycle)		Relative responses to 0 5 μg histamine + 50 ng bradykinin			
			2nd dose cycle		3rd dose cycle	
	Increase in pulm art pressure (cm H_2O)	Decrease in tracheal pressure ampl (%)	Increase in pulm art pressure	Decrease in tracheal pressure ampl	Increase in pulm art pressure	Decrea in trachea pressur ampl
—	2 5 $\pm$ 0 8	13 7 $\pm$ 3 6	1 7	2 6	2 1	5 8
Phenylbutazone	1 2 $\pm$ 0 7	25 7 $\pm$ 9 5	2 7	2 1	0 2	0 2
Phenacetin	1 4 $\pm$ 0 6	15 3 $\pm$ 7 8	1 2	4 3	1 4	3 1
Estriol	0 4 $\pm$ 0 7	9 2 $\pm$ 3 4	5 0	5 2	5 0	4 8

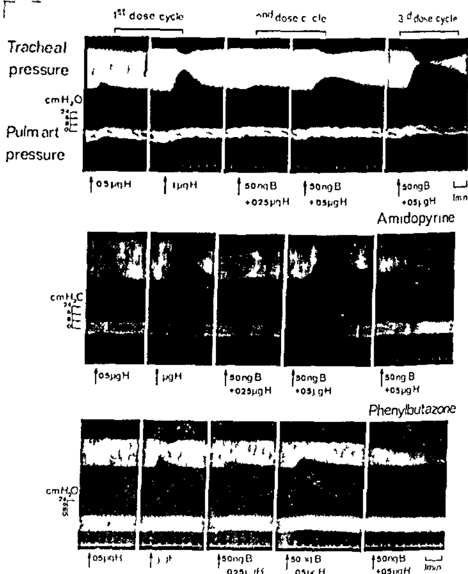


Fig 8

Isolated guinea pig lung preparations Upper section Control experiment comparison of the responses to 0 5 and 1 0 μg of histamine (H) in the 1st dose cycle with those to 0 25 and 0 5 μg H added immediately after 50 ng of bradykinin (B) in the 2nd dose cycle 20 min after changing the perfusion fluid from one stock of Krebs Ringer solution to another stock 0 5 μg of H with 50 ng of B was again administered (3rd dose cycle) Note the strong decrease in the tracheal pressure amplitude in the 3rd cycle Note also that the amplitude does not return to normal Middle section Inhibitory effect of amidopyrine, 20 μg per ml added to the perfusion fluid between the 2nd and 3rd cycle on the responses to H in combination with B Lower section Inhibitory effect of phenylbutazone 20 μg per ml added to the perfusion fluid between the 2nd and 3rd cycle on the responses to H in combination with B

amplitude to normal values in the control periods of the third or fourth dose cycles This idea seems to be supported by the following findings After repeated administration of 5 hydroxytryptamine the effect on the tracheal pressure amplitude decreased instead of increasing Furthermore the amplitude remained unchanged in the intervals between the doses These effects which are at variance with those of histamine and bradykinin correspond well with the small effect of 5 hydroxytryptamine on the vascular permeability in guinea pigs (WILHELM 1962)

A dose of bradykinin which was about twenty times smaller than the liminal dose effective on the tracheal and the pulmonary arterial pressure enhanced significantly both the decrease in the tracheal pressure amplitude and the increase in the pulmonary arterial pressure induced by histamine This effect of low-doses of bradykinin may be explained by assuming that bradykinin increases the vascular permeability for histamine WILHELM (1962) showed that especially synthetic bradykinin increases vascular permeability *at very low concentrations*

COTRAN and MAJNO (1964) distinguished four types of pathological vascular leakage One of them is the histamine type which would be induced by histamine 5 hydroxytryptamine and bradykinin These agents elicit the early transient type of inflammation reaction The essential morphological features of this type of vascular leakage are now well known COTRAN and MAJNO (1964) found that the leaking vessels were predominantly venules and that the cellular mechanism was a partial disconnection of the endothelial cells along their junctions This means that pores open between the cells while the basement membrane remains intact resisting the passage of plasma proteins but not that of fluid The permeability for fluid and for plasma proteins are supposedly two different mechanisms (WINTER 1966) The vascular leakage starts immediately upon local appearance of the mediator e g histamine and lasts for a short period in the order of 30 min (COTRAN and MAJNO 1964) The effects on the tracheal pressure amplitude observed in our experiments in which histamine and bradykinin added repeatedly can be explained by assuming a cumulative effect of the successive administrations on the vascular permeability Thus the increased permeability may enable the passage of increasing amounts of the drugs into the interstitial fluid This could

an agent with a small anti-inflammatory activity and estriol as a representative of the steroid anti-inflammatory agents which supposedly fail to inhibit the immediate short term response to injury such as erythema and oedema in the guinea pig (WHITEHOUSE, 1965). The results of these experiments are summarized in Table 3. This Table shows that phenylbutazone inhibited almost completely both the decrease in tracheal pressure amplitude and the increase in pulmonary arterial pressure induced by the combined administration of histamine and bradykinin in the third dose cycle. Phenacetin had a much smaller effect and estriol showed no effect at all.

4. DISCUSSION

In the present experiments histamine and bradykinin appeared to have two common effects on the amplitude of the tracheal pressure. In the first place both substances caused the amplitude to decrease. The magnitude of this response as well as its duration increased with successive administrations at 10 min interval. Secondly the amplitude of the tracheal pressure did not return to its normal value after repeated administration. This latter phenomenon was more pronounced for bradykinin than for histamine.

Whereas histamine had a very pronounced effect on the tracheal pressure changes and a less pronounced effect on the pulmonary arterial pressure, bradykinin in the doses used had a small effect on the tracheal pressure changes and a more pronounced effect on the pulmonary arterial pressure. The effect of bradykinin on the arterial pressure increased with the dose cycle whereas the effect of histamine on the arterial pressure did not change significantly during the 4 dose cycles.

It was found that alternation of histamine and bradykinin in doses which were active by themselves soon caused a blockage of the airways by the accumulation of fluid. Histamine and especially synthetic bradykinin are highly potent agents in increasing the vascular permeability in guinea pigs (WILHELM, 1962). This increased vascular permeability accompanied by an accumulation of fluid in the interstitial spaces could explain the increase in effect on the tracheal pressure with successive administrations of either histamine or bradykinin as well as the incomplete return of the

In conclusion it should be emphasized that the described effect of the anti inflammatory agents may be considered as a relatively unspecific effect on the vascular endothelium

ACKNOWLEDGEMENT

It is a pleasure to thank Prof Dr C van der Meer for his advice and helpful criticism during the investigation and during the preparation of this manuscript

SUMMARY

In isolated guinea pig lungs with vascular perfusion, histamine, bradykinin and 5 hydroxytryptamine caused a decrease in the tracheal pressure amplitude and an increase in the pulmonary arterial pressure Whereas histamine had a very pronounced effect on the tracheal pressure changes and a less pronounced effect on the pulmonary arterial pressure, bradykinin had *only* a small effect on the tracheal pressure changes and a more pronounced effect on the pulmonary arterial pressure

On repeated administration of histamine and bradykinin the effect on the tracheal pressure amplitude increased and furthermore, the amplitude did not return to its normal value These phenomena were ascribed to increased vascular permeability This idea seems to be supported by the findings that 1 the phenomena were not found after successive administrations of 5 hydroxytryptamine 2 a subliminal dose of bradykinin significantly increased both the decrease in the tracheal pressure amplitude and the increase in the pulmonary arterial pressure induced by histamine

The non steroid anti inflammatory agents phenylbutazone amidopyrine, phenazone and sodium salicylate inhibited to a varying extent the decrease in tracheal pressure amplitude induced by histamine or by histamine preceded by a subliminal dose of bradykinin In this last case the increase in pulmonary arterial pressure was also inhibited Phenacetin had a slight effect, whereas estriol a steroid anti inflammatory agent, had no effect at all

It is concluded that the described effect of the non steroid anti inflammatory agents may be considered as a relatively unspecific effect on the vascular endothelium

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result in a stronger contraction of the bronchial smooth muscles As a matter of course the amount of interstitial fluid would also increase thus decreasing the compliance of the lungs Since the sinusoidal changes in tracheal pressure are the result of the sinus oidal pressure changes produced outside the lung the amplitude of the pressure changes in the trachea depend on both the airway resistance and the lung compliance The compliance will decrease as a result of an increase in the amount of interstitial fluid This increase may be the explanation for the incomplete recovery of the amplitude after repeated administration of either histamine or bradykinin

In the present set up the non steroid anti inflammatory agents phenylbutazone amidopyrine phenazone and sodium salicylate inhibited to varying degrees the decrease in tracheal pressure amplitude induced by histamine or by histamine preceded by a subliminal dose of bradykinin

Phenacetin had a slight effect whereas estriol a steroid anti inflammatory agent had no effect at all Some parallelism was found between the inhibitory activities of the non steroid anti inflammatory agents and their potencies for suppressing skin ery thema caused by ultra violet irradiation in guinea pigs (WINDLER *et al* 1958) These findings may also support the assumption that the increase in effect after successive administrations is due to an increased vascular permeability

It is noteworthy that the increase in pulmonary arterial pressure induced by histamine in combination with bradykinin was also inhibited by the non steroid anti inflammatory agents investigated (Table 3) However a similar effect caused by giving a third and fourth dose of histamine alone did not seem to be affected by these agents (Fig 7)

In previous experiments (AARSEN 1966) no effect of the non steroid anti inflammatory agents on the responses to histamine or bradykinin could be demonstrated The explanation may be that in these experiments the lung vessels were perfused at a slower rate and that the dose of both histamine and bradykinin were higher This caused persistent occlusion of the vessels which could only be overcome by temporarily increasing the perfusion pressure This procedure might conceivably have caused irreversible damage to the vascular walls

In conclusion it should be emphasized that the described effect of the anti inflammatory agents may be considered as a relatively unspecific effect on the vascular endothelium

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In the present set up the non steroid anti inflammatory agents phenylbutazone, *amidopyrine, phenazone and sodium* salicylate inhibited to varying degrees the decrease in tracheal pressure amplitude induced by histamine or by histamine preceded by a *subliminal dose of bradykinin*.

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DESENSITIZATION OF THE END PLATE MEMBRANE FOLLOWING CHOLINESTERASE INHIBITION, AN ADJUSTMENT TO A NEW WORKING SITUATION

BY

E MEETER

I regard it as a great honour to have been asked to contribute to this series of lectures given to commemorate the founding of the first chair in pharmacology in this country, sixty years ago It forced me to look back on my work of the past 15 years in order to select a subject of sufficient general interest I decided upon a contribution dealing with the adjustments occurring in the post-synaptic membrane of the motor end plate when it is subjected to variations in the concentration of depolarizing agents in its vicinity

The reason for investigating this subject is its usefulness in the understanding of the mechanism of action of anticholinesterase compounds a subject of importance in our laboratory Recently Dr Wolthuis and I (MEETER and WOLTHUIS, 1968) have discussed the processes which play a part in the spontaneous recovery of respiration and neuromuscular transmission in the rat after *poisoning* with various anticholinesterases Spontaneous recovery may be produced by three different mechanisms 1 spontaneous reactivation of "old enzyme, when the inhibitor is *relatively* loosely bound 2 synthesis of new enzyme, a slow process which plays little or no part in recovery during the first 12 h, and 3 "adaptation" a process which can be demonstrated in the myoneural junction of the rat when it has been poisoned with a firmly bound inhibitor like DFP This adaptation will be the main subject of the present contribution

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iodide). Before, and at regular intervals after the injection the neuromuscular transmission was tested by stimulating the sciatic nerve with trains of pulses of 100 μsec at frequencies of 25, 50, 100 and 200/sec, each train lasting 3 sec. The contractions of the gastrocnemius soleus muscles were recorded. Between the tests the nerve was stimulated with 1 pulse per 10 sec throughout the experiment. It appeared that shortly after the S54 injection the muscles were unable to produce the normal tetanic contractions, instead they showed little more than a single twitch for each train of stimuli. In the course of the subsequent hours the ability to sustain a tetanic contraction partly returned, at least at the lower frequencies. A second injection of the same dose of S54 then produced little, if any effect on the response of the muscles which shows that the partial recovery had not been due to an increasing availability of functional cholinesterase, but to some kind of adaptation of the neuromuscular junction to the conditions created by the blocked enzyme.

Fig. 1 also shows that about 3 h after the first S54 injection no further improvement of the transmission took place, apparently the adaptation had come to an end. Similar results were obtained in previously published DFP-experiments (MEETER and WOLTHUIS, 1968).

THE ANTICHOLINESTERASE POISONED, ISOLATED RAT DIAPHRAGM

One way to investigate this limited, but for the purposes of therapy rather important recovery mechanism, is to study isolated rat nerve muscle preparations in a situation comparable to that of the rat muscles *in situ*, i.e. under a continuous regime of indirect stimuli. Some years ago (MEETER, 1958) it was found that during continuous indirect stimulation of the isolated rat phrenic nerve-diaphragm preparation DFP administration in the bath initially caused a depolarization of the end plate region. This depolarization reached a maximum after an interval depending on the dose of DFP (1 h with 0.33 mg/l and ½ h after 0.66 mg/l), following which the depolarization slowly subsided.

More recently, this investigation was taken up again in order to study the reason for the waning of the depolarization. In these experiments the depolarization of the end plate region was measured

THE INTACT, ANTICHOLINESTERASE POISONED RAT

In Fig 1 the results are shown of an experiment in which an anaesthetized (barbital 215 mg/kg, i p), atropinized (50 mg/kg i p) artificially ventilated rat was injected with a lethal dose (0 1 mg/kg, i v) of the irreversible cholinesterase inhibitor S54 (cyclopentyl S 2 trimethyl ammonioethyl methylphosphonothioate

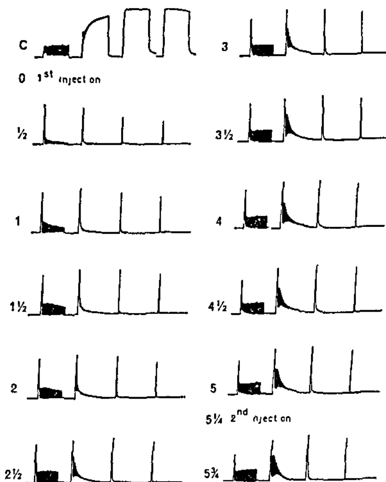


Fig 1

Traces showing tetanic contractions of the gastrocnemius soleus muscles in a rat, evoked by stimulating the sciatic nerve with 3 sec trains of pulses at 25 50, 100 and 200/sec C = control responses At 0 an i v injection of 0 1 mg/kg of the irreversible cholinesterase inhibitor S54 The figures on the left of each group of traces denote the time in hours after the injection At 5¼ h a second equal injection of S54 was given

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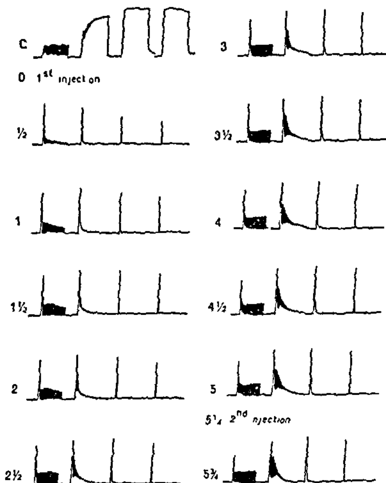


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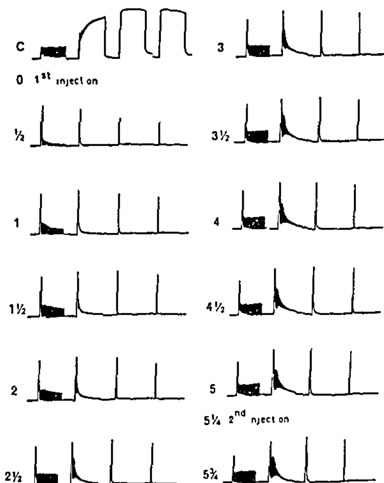


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DFP, the potential measured at the end plates is affected very little by indirect stimulation Soon after the addition of DFP (final concentration 6 6 mg/l) the records obtained during rest begin to indicate a depolarization which reaches a maximum of 1 56 ± 0 10 mV in 8 to 12 min (12 experiments) and then slowly wanes with a half-decay time of 15 to 20 min The depolarization found at the end of a period of stimulation was always higher than after the corresponding resting period, the maximum reached being 3 10 ± 0 16 mV As mentioned before, in these experiments the potential difference between the top end of the muscle and the end plate region was measured In an ideally dissected muscle strip this potential difference should be zero during rest However, in practice demarcation potentials of damaged fibres produce a potential difference which has nothing to do with the events occurring at the end plates Usually these demarcation potentials slowly decrease in the course of the experiment The rate of this decrease cannot be determined after the addition of a drug with an irreversible action on the end plates like DFP Therefore, the longer the time after DFP had been added, the more uncertain the meaning of the absolute potentials measured became For this reason most attention was paid to the ' difference potentials" obtained by subtracting the potential after rest from that found following a period of nerve stimulation These difference potentials can be regarded as the contribution to the depolarization made by the indirect stimulation of the muscle

During the period in which the depolarization decreased in these experiments the preparations were treated with a test-dose of acetylcholine chloride (ACh) three times each time for 3 *min*, i e the shortest possible time which allowed a measurement of a potential to be made This procedure, performed with ACh at a final concentration of 2 8 μ mole/l at 28 58 and 108 min after the addition of the DFP to the bath provided us with information about the ACh sensitivity of the postsynaptic membrane during the course of the events The resulting test responses i e the extra depolarizations *due to the added ACh, were 1 50 ± 0 09, 0 82 ± 0 10* and 0 57 ± 0 09 mV respectively

In Fig 3 the averages of the difference potentials from 7 experiments have been plotted against the time after DFP administration From a value of 1 25 ± 0 12 mV, 12½ min after DFP addition,

with the method described by FATT (1950), which was also used in the earlier experiments (MEETER, 1958) A strip of muscle with the nerve attached was placed vertically in a bath filled with oxygenated Krebs Ringer solution When the potentials on the surface of the muscle had to be measured, the fluid was allowed to run out of the bath Its meniscus then served as an electrode which sweeped along the length of the diaphragm strip The other electrode was placed at the top end of the muscle The potential at the end plate region, about half way along the muscle, was noted The procedure was to stimulate the nerve for 2 5 min with 1 stimulus per 2 sec with supramaximal pulses of 0 1 msec, and subsequently to allow it to rest for 2 5 min At the end of each 2 5 minute period the potential at the end-plate region was measured

An example of the results obtained, given in Fig 2, shows that in the control period, i e before treatment of the muscle with

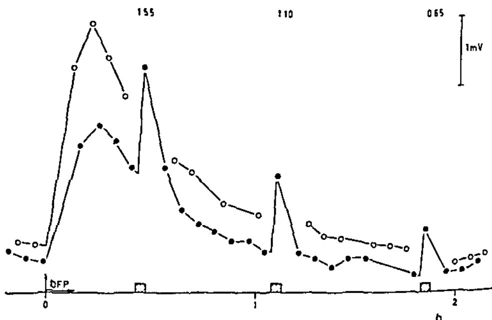


Fig 2

Potentials found at the end plate region of a strip of rat diaphragm The line o—o shows the potentials found after 2 5 min of indirect stimulation the line •—• represents the potentials obtained in the same muscle, each time after 2 5 min of rest The hatched rectangles indicate the periods that the muscles were given a test dose of ACh (2 8 μM) in the bath The figures on top present the amplitudes in mV of the extra depolarizations evoked by these test doses.

the end plate membrane to this substance Probably the desensitization observed in the present experiments was also caused by ACh being present in abnormally high concentration in the vicinity of the end plate due to the cholinesterase inhibition If this explanation is correct, it should be possible to reduce the rapidity of the decrease of the difference potential by allowing the preparation a suitable period of rest In a series of 9 experiments which were essentially performed in the same way as the previous ones, 5 preparations were allowed a period of rest lasting from 28 to 78 min after the addition of DFP (final concentration 3 3 mg/l), whereas the remaining 4 preparations were indirectly stimulated as usual, without a prolonged interruption No ACh test doses were given The 5 experimental muscles which underwent the period of rest, and the 4 control ones responded in a reasonably similar way to the DFP their difference potentials, found at the earliest possible moment after DFP administration when these potentials could reliably be determined (after 17½ min) were 1 45–2 00 mV (average 1 90) and 1 28–2 20 mV (average 1 65), respectively In contrast to this similarity, the first difference potentials noted following the period of rest in the 5 experimental muscles were about twice as high as the corresponding values recorded for the 4 control preparations 0 73–1 45 mV (average 1 16) against 0 28–0 65 mV (average 0 48), a significant difference according to the Wilcoxon Mann Whitney rank sum test ($p_2 < 0\ 05$)

THE UNPOISONED, ISOLATED RAT DIAPHRAGM

In order to compare the rate of desensitization of the end plates in the isolated rat diaphragm with that found in the muscles of the intact rat experiments were carried out in which known concentrations of ACh were maintained in the bath for periods lasting many hours At regular intervals ACh test doses were applied in addition to the continuously present concentration to determine the sensitivity of the postsynaptic membrane These test doses were present in the bath for 6 min only The muscles were not treated with DFP in order to avoid the "spontaneous" depolarization which is seen after DFP administration, even in the resting muscle Since the cholinesterase in these muscles was not inhibited a much higher concentration of ACh (0 56 mM) was needed than in the previous

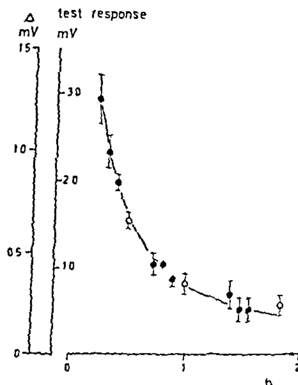


Fig 3

Difference potentials (•) plotted against the time after DFP injection averages from 7 experiments On the same abscissa the responses to ACh test doses (O) are plotted The vertical bars represent 2 × S E of mean Ordinate for the difference potentials indicated by Δ mV

the difference potential decreased to 0 22 ± 0 06 mV in 80 min In the same figure the amplitudes of the responses to the test doses of ACh have been plotted The scale on the ordinate used for plotting these test responses was chosen in such a way that the value of the second response fell on the graph representing the difference potentials It is clear that if this is done the values for the first and the third responses fit remarkably well on that same graph This indicates that the rapidity of the decrease of the sensitivity of the end plate membrane for ACh is almost exactly the same as that of the decrease of the difference potentials It is therefore very likely that the decreasing ACh sensitivity is the cause of the decreasing depolarization

Some years ago THESLEFF (1955a and b) and KATZ and THESLEFF (1957) showed that the continuous presence of ACh desensitizes

the end plate membrane to this substance Probably the desensitization observed in the present experiments was also caused by ACh being present in abnormally high concentration in the vicinity of the end plate due to the cholinesterase inhibition If this explanation is correct, it should be possible to reduce the rapidity of the decrease of the difference potential by allowing the preparation a suitable period of rest In a series of 9 experiments, which were essentially performed in the same way as the previous ones, 5 preparations were allowed a period of rest lasting from 28 to 78 min after the addition of DFP (final concentration 3 3 mg/l), whereas the remaining 4 preparations were indirectly stimulated as usual without a prolonged interruption No ACh test-doses were given The 5 experimental muscles which underwent the period of rest, and the 4 control ones responded in a reasonably similar way to the DFP their difference potentials found at the earliest possible moment after DFP administration when these potentials could reliably be determined (after 17½ min), were 1 45–2 00 mV (average 1 90) and 1 28–2 20 mV (average 1 65), respectively In contrast to this similarity the first difference potentials noted following the period of rest in the 5 experimental muscles were about *twice as high as* the corresponding values recorded for the 4 control preparations 0 73–1 45 mV (average 1 16) against 0 28–0 65 mV (average 0 48), a significant difference according to the Wilcoxon, Mann Whitney rank sum test ($p_2 < 0\ 05$)

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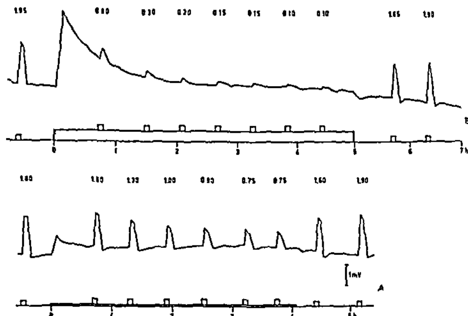


Fig 4

Potentials measured at the end plate region From time 0 onwards the bath contained a certain concentration of ACh 0 22 mM in A and 1 12 mM in B, indicated by the elongated rectangles at the bottom At regular intervals test doses of ACh (0 56 mM) were given indicated by small rectangles The figures on top indicate the amplitudes of the responses to the test doses in mV

experiments, to evoke a suitable test response The preparations were not stimulated electrically

Fig 4 shows two examples of the results obtained After a few control responses to ACh had been obtained (only the final one is shown in the figure), the continuous ACh treatment was started Immediately a depolarization of the end plate region occurred which then gradually subsided The responses to the test doses showed that the sensitivity slowly decreased When 4 or 5 h later, the ACh exposure was stopped the sensitivity rapidly returned to the control value In these experiments it was found that the absolute value of the control test response obtained before the start of the prolonged ACh exposure was highly reproducible from muscle to muscle The average from 9 experiments was 1 85 ± 0 08 mV Likewise, the test responses recorded at the end of the experiment, when the preparation had been in the bath for 6 to 8 h, and had fully recovered from the prolonged exposure to ACh,

were remarkably similar to those at the start 1 89 ± 0 09 mV in the same experiments

The amount of reduction of the test response which was ultimately caused by the continuous presence of ACh is shown in Fig 5, plotted against the ACh concentration used The reduction is expressed as a percentage of the average values of the controls taken before and after the ACh treatment It appears that the relationship between the percentage reduction and the log ACh concentration is linear over the major part of the concentration range However, at the lower concentrations this linearity is lost, and it seems that a threshold concentration has to be reached before the mechanism of desensitization comes into operation Also shown in Fig 5 is the time which elapsed from the start of

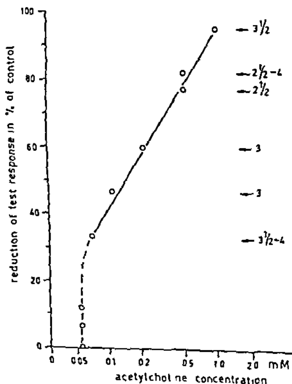


Fig 5

Relationship between ACh concentration and amount of desensitization at the end of the process, expressed as a percentage reduction of the test response

the ACh treatment to the (approximate) moment after which no further reduction of the test responses was seen. It appeared that irrespective of the ACh concentration used the desensitization process always took about 3 h. In contrast to this slow development of the desensitization is the much higher rate with which the original sensitivity was restored when the preparation was returned to ACh free medium. Although the accuracy of the observations made on this point is rather limited it became clear that the original sensitivity was restored within 1½ h at the most.

In previous unpublished experiments it was found that if one rat diaphragm strip is tested with different doses of ACh the evoked initial depolarization is linearly related to the ACh concentration. In Fig. 6 the initial depolarizations observed in the experiments of Fig. 5 are plotted against the applied ACh concentration. Not only the initial depolarizations produced by the

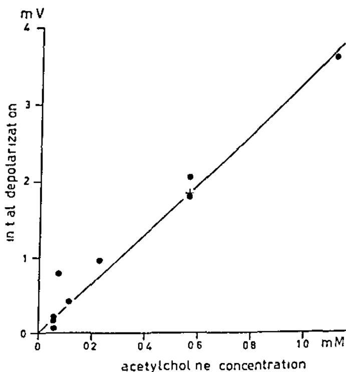


Fig. 6

Relationship between ACh concentration and initial depolarization in the experiments of Fig. 5. A straight line is drawn through the average of the control test responses (+) and the origin.

prolonged ACh treatment, but also the control test responses were plotted As mentioned above, these control test responses were very similar for all muscles, therefore only their average is represented in Fig 6 A straight line drawn though this point and the origin shows a reasonably good fit with the other points in the graph, indicating that the expected linear relationship between ACh concentration and initial depolarization also existed in these experiments With the aid of this line in Fig 6, it is possible to 'translate' the log ACh concentration on the abscissa of the graph in Fig 5 into log initial depolarization This, in turn proves that over the major part of the range the desensitization is linearly related to the logarithm of the amplitude of the *initial* depolarization

THE EFFECT OF CALCIUM ON THE DESENSITIZATION

Recently Manthey (see NASTUK, 1966) has studied the effects of the ionic composition of the perfusion medium on the desensitization process in the frog muscle He measured the membrane resistance and the depolarization at the myoneural junction and determined the effect of perfusion of the muscle with carbamyl choline for periods of 6 to 8 min It was observed that a reduction of the calcium content of the medium from 1 8 mM to zero, markedly decreased the rate of desensitization, whereas in 10 mM Ca medium the process was accelerated This desensitization was also found to be dependent on the sodium concentration reduction of the Na content from 120 to 28 mM definitely increased the normal rate (at 1 8 mM Ca) but had little effect on the already accelerated desensitization in the presence of 10 mM calcium

A preliminary investigation of the effect of calcium on the rate of desensitization in the rat diaphragm was carried out With the aid of the Fatt method we recorded the depolarization of the end-plate region caused by exposure of the muscle to an ACh concentration of 0 50 mM for 15 or 60 *min* In 4 experiments comparisons were made between the rates of desensitization in Krebs-Ringer solutions containing either zero, 2 5 (normal value) or 5 0 mM Ca No dramatic differences were observed but, in contrast to the frog muscle, the rat end plate increased its rapidity of desensitization in Ca free medium and decreased it slightly in the

presence of the doubled calcium concentration EDTA was not used in the Ca free solution A further increase in calcium content in the normally used bicarbonate buffered Krebs Ringer solution is not feasible because above 5 mM a turbidity develops as a result of calcium carbonate formation

DISCUSSION

The present experiments have confirmed the findings of MEETER and WOLTHUIS (1968) that in the rat poisoned with an anticholinesterase inhibitor, the transmission in the motor end plate partly recovers in the presence of an undiminished inhibition of the enzyme This process progressed up to a limit which was reached about 3 h after the administration of the anticholinesterase The investigations in the isolated phrenic nerve diaphragm preparation confirmed earlier findings (MEETER, 1958) that in the anticholinesterase treated, indirectly stimulated muscle initially a depolarization ensued, which subsequently waned In the experiments on this point, described here, the depolarization was also measured after a period of rest By subtracting these "resting depolarizations" from the depolarizations found after stimulation, "difference potentials" were obtained which to some extent represent the contribution of the indirect stimulation to the end plate depolarization The difference potentials also decreased during the course of an experiment Moreover the ACh sensitivity of the post synaptic membrane in these muscles was found to go down at the same rate as the difference potentials In similar experiments a significantly smaller decrease of the difference potential was observed when the muscles were allowed a 50 min period without indirect stimulation

These results obtained in the isolated active, DFP treated muscles suggest that the decrease of the depolarization of the end plate region is caused by a decrease of the sensitivity of the postsynaptic membrane to ACh Moreover, the rate of this decrease is slowed if the activity of the muscle is interrupted which suggests that the amount of ACh present in the vicinity of the end plates determines the rapidity of this process This was tested in unpoisoned muscles exposed to various ACh concentrations for a number of hours It was indeed found, that the rate of desensiti

zation depends on the ACh concentration However, the time needed to reach the end point was remarkably independent of the amount of ACh present, always being about 3 h This may indicate a similarity to the adaptation process observed in the intact poisoned rat which also reached its end point in approximately 3 h

As discussed by THESLEF and QUASTEL (1965) the loss of the ability of the anticholinesterase poisoned muscle to sustain a tetanic contraction is believed to be caused by rapid desensitization of the end plate membrane due to the large amounts of ACh present after the arrival of the first nerve volleys This contention is mainly based on the extremely rapid desensitization observed by KATZ and THESLEFF (1957) after iontophoretic application of ACh These authors observed that the end plates desensitized in a few seconds, with a rate depending on the initial depolarization It should be kept in mind that the initial depolarization does not only depend on the amount of ACh released by the nerve endings but also on the pre-existing sensitivity Consequently, a lowered base line sensitivity will cause a lower rate of desensitization resulting in a better sustained tetanic response KATZ and THESLEFF (1957) wondered whether the fast desensitization seen in iontophoresis experiments and the slow desensitization observed after bath application of ACh (FATT, 1950, THESLEFF 1955a and b) are actually one process with a fast start and a slow "tail" Although the present experiments produced no further information on this point they show that in the rat the "end of the tail" occurs in about 3 h

If it is true that the adaptation in the poisoned animal is due to the desensitization of the postsynaptic membrane to ACh, other means of desensitizing the membrane should also improve trans mission In fact Wills and co workers (see WILLS, 1963) have studied the effectiveness of a great many competitive antagonists to ACh in restoring neuromuscular transmission in the poisoned animal They obtained positive results in many cases Recently RUMP and KALISZAN (1968) investigated the effect of curare like drugs on the rate [illegible]

[illegible]

were administered Their results show that the dose range which produces this curative effect is very limited. This might be expected

for the sensitivity of the already desensitized postsynaptic membrane is easily lowered too far

KARCZMAR, KOKETSU and SOEDA (1968) have just published results which demonstrate a restorative effect of methoxyambenonium and *d–TC* on the tetanic response of the indirectly stimulated, DFP or TEPP treated frog sartorius muscle They ascribe this *effect to an enzyme reactivating action of these drugs, because* the beneficial effect remained even after prolonged washing of the *preparations, that is, when no more methoxyambenonium or* d–TC could be present However, the crucial test, whether a renewed exposure to *enzyme inhibitor again* produces the expected loss of tetanic response, was not done So far it appears more likely that what the authors have seen was the result of an increased desensitization of the end-plate membrane

It might be argued that, since cholinesterase reactivating oximes are available for the treatment of anticholinesterase poisoning, end-plate desensitizing drugs are of no practical importance However, a number of compounds are known to produce an oxime resistant inhibition (see MEETER and WOLTHUIS, 1968) In the case of a poisoning with such an inhibitor even a small improvement of neuromuscular transmission would be helpful. Because of their limited useful dose range, the competitive ACh antagonists cannot be safely applied, as even a slight overdose would aggravate the paralysis What is needed is a method of *increasing* the rate of development of the desensitization without interfering *with the end-point That such a thing might be feasible is suggested* by the few reports which describe rate changes JENKINSON, STAMENOVIĆ and WHITAKER *(1968) present* indirect evidence indicating that noradrenaline reduces the rate of desensitization of the frog end-plate in the presence of ACh Moreover, they mention preliminary, unpublished results of Stamenovic showing a reduction of the rate by both adrenaline and noradrenaline Although these rate changes are not in the right direction for useful therapy, they may indicate that the rate can be changed The observations of Manthey (see NASTUK, 1966) concerning the rate increasing effect of a high calcium level in the medium also stresses this point Unfortunately *the relatively small change in Ca^{++} level which* would be feasible in an intact animal probably has little effect

A point of fundamental interest is the observation made in the

present experiments that, although the end point reached by the desensitization is linearly related to the log ACh concentration and to the log initial depolarization, below a certain level this is no longer true A threshold concentration of ACh seems to be needed This result is in agreement with the findings of GISSEN and NASTUK (1966) in the frog sartorius muscle They observed that carbamylcholine at a concentration of 0 11 mM produced a sustained depolarization for 2 h, whereas higher concentrations (0 27 mM and more) gave an initial depolarization followed by a repolarization It looks as if there is a range of (low) concentrations of depolarizing compounds which cause no desensitization

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr O L Wolthuis, without his active co-operation part of this work would not have been possible, to Dr R L Polak for his very stimulating interest in this work, and to Mr A Beyersbergen-van Henegouwen for his unfailing technical assistance

SUMMARY

A survey is given of the work done in this laboratory concerning spontaneous changes in the postsynaptic membrane of the rat end plate after poisoning with an antichohnesterase agent Partial spontaneous recovery of the ability to sustain a tetanic contraction was observed in the indirectly stimulated muscles of the intact rat poisoned with an irreversible cholinesterase inhibitor This process of 'adaptation reached an end point after about 3 h The isolated indirectly stimulated phrenic nerve diaphragm preparation (pndp) poisoned with DFP, showed a depolarization of the end plate area which slowly subsided Parallel with this decrease of depolarization the sensitivity of the end plate membrane to acetylcholine, ACh decreased This decrease was slowed when the indirect stimulation was stopped by 50 min In the isolated unstimulated, unpoisoned pndp the sensitivity of the end plate membrane decreased in the presence of ACh The end point of this desensitization was reached in about 3 h These results suggest that the adaptation of the neuromuscular junction in the intact poisoned rat is caused by the desensitization of its end plates due to the large amount of ACh present in their vicinity The ultimate desensitization reached in the unpoisoned pndp was found to be linearly related to the log ACh concentration between 0 07 and 1 1 mM, as well as to the log initial depolarization caused by the ACh Below 0 07 mM irregularities were seen which may suggest that at small initial depolarizations no desensitization occurs

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CHOLINO SENSITIVE SIDES OF NERVE- AND MUSCLE MEMBRANE

BY

A DEN HERTOG AND W LAMMERS

Dr Meeter's paper is concerned with the sensitivity of acetylcholine receptors in the end plate region During recent years we have been interested in the occurrence of such cholino sensitive sites outside this end plate region It is a well known fact that after denervation of striated muscles such receptors "appear" along the whole muscle fibre It seems a strange phenomenon that these receptors are non-existent in normal muscles, whereas after denervation, acetylcholine sensitivity appears as a new property It is known that such acetylcholine receptors are present in mammalian C fibres It appeared from experiments by ARMETT and RITCHIE (1960, 1961) and recent experiments by DEN HERTOG and RITCHIE (1968) that just as in the end plate, acetylcholine induces depolarization of these C fibres which is caused mainly by an increase in the sodium permeability of the membrane Tubocurarine (TC) is a blocking agent for these receptors They have no function whatever in the conduction of the action potential along the nerve fibres

Because of these facts, it seemed worthwhile to look for such cholino-sensitive receptors on the striated muscle membrane outside the end plate region In our experiments a technique was used in which a micro-electrode was placed in the muscle fibres of the tibialis anterior muscle of the cat This micro-electrode was used as a stimulating and recording electrode (DEN HERTOG, 1966) This makes it possible to register electrical phenomena in normal muscle *in vivo* (DEN HERTOG, LAMMERS, VAN DER MOST VAN SPIJK and RAS, 1966)

Succinylcholine (S Ch) was used as a cholinergic substance

because it causes long lasting depolarization in contrast to acetyl choline From our experiments it appeared that S Ch in a totally paralyzing dose induces a depolarization of the muscle membrane and it will be shown that this effect is not restricted to a particular part of the fibre This was already indicated by PATON and ZAIMIS (1949) but it was assumed that the point of attack of S Ch was in the end plate and that the resulting depolarization subsequently spread along the muscle fibre The time course of this phenomenon might show whether this explanation is true for the depolarization we saw However we were not able to measure the depolarization immediately after injecting of S Ch because of the subsequent fibrillations these fibrillations will break the micro electrodes We tried to overcome this difficulty by placing two micro electrodes in one fibre and measuring the time course of the depolarization in this manner This is a rather complicated method with a high percentage of failures in these *in two* experiments But in the positive trials we observed that the depolarizations recorded through both electrodes were always simultaneous Additional proof that the choline sensitivity is not located in the end plate was given by the following experiments Two electrically isolated parts of the muscle fibres were created by heating the muscle locally In the proximal part of the fibres an indirect action potential could be elicited in about 95 % of the fibres whereas this indirect action potential occurred in only about 5 % of the fibres on the other side of the gap Because of this and because double innervation of mammalian striated muscle fibres is rare we assume that an end plate free part of the muscle fibre had been created From our experiments it appeared that on both sides of such an artificial gap S Ch induces a depolarization which is of the same magnitude and which follows the same time course

This action of S Ch can not be blocked by a dose of TC that just paralyzes the muscle completely for indirect stimulation However if a higher dose of TC is used (2 mg/kg) this depolarizing action of S Ch is inhibited In these instances the normal propagation of the action potential was not affected this was measured by insertion of two electrodes as much as 10 mm apart into the same fibre

Thus it can be concluded that S Ch initiates a depolarization

along the whole muscle fibre and that this depolarization does not originate at the end plate We therefore must assume (succinyl) choline sensitive sites outside the end plate region which can be blocked by TC This agrees with the results of BROWN, PATON and VIANNE DIAS (1949), JARCHO, EYZAGUIRRE, TALBOT and LILIENTHAL jr (1950), JEWELL and ZAIMIS (1954) and PORTELA, PEREZ, STEWART, LUCHELLI and STRAJMAN (1964a, b), all of whose experiments indicated the existence of cholino sensitive sites along the whole muscle fibre membrane This suggestion is also supported by the results of studies with labelled depolarizing drugs, published by TAYLOR, CREESE, NEDERGAARD and CASE (1965), and by WASER (1960) Thus TAYLOR *et al*, found in their experiments with labelled decamethonium that, although the peak uptake of radioactivity was located at the nerve ending, there was a definite uptake in the region outside the end plate The uptake of carbachol was uniform along the muscle membrane Both the uptake of decamethonium and of carbachol were diminished by TC Such a cholino sensitivity along the fibre membrane as found in our mammalian fibres, was not (or barely) detectable outside the end plate region in the frog sartorius muscle (KATZ and MILEDI, 1961, MILEDI, 1960, OCHS, 1966) This difference might be due to the difference in muscle temperature (37 °C in our experiments and about 20 °C in the frog muscle) to the fact that our experiments were in *vivo* whereas those of the frog were in *vitro* or to differences in the concentration of the drug added Furthermore PORTELA *et al* (1964a, b) have argued that cholino sensitive receptors do indeed exist along the whole muscle fibre but that under *in vitro* circumstances the [illegible]

[illegible] Although our results seem to indicate the presence of cholino sensitive sites along the muscle membrane this does not mean that acetylcholine has a physiological role in the conduction of the action potential as assumed by PORTELA *et al* (1964a, b) Indeed the reverse seems to be suggested since we were able to block the sensitivity of the receptors to S Ch by TC without affecting conduction of the directly elicited action potentials In this respect therefore, the membranes of skeletal muscle fibres resemble those of C fibres

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STUDIES ON THE MECHANISM OF INDUCED CHANGES IN THE POTASSIUM BALANCE OF THE HEART [1]

BY

E D GERLINGS AND J P GILMORE [2]

I INTRODUCTION

Since the experiments of RINGER in 1883 it has been well established that changes in the intracellular content and/or distribution of certain ions play an important role in modulating myocardial contractility While considerable emphasis has been given recently to the calcium ion, the studies of both HAJDU (1953) and SARNOFF and associates (1963) have shown that under a variety of experimental conditions, changes in myocardial performance are associated with changes in net potassium balance The former has demonstrated in the frog heart that the increase in contractility associated with increasing heart rate is associated with proportional decreases in myocardial potassium (RINGER, 1883) SARNOFF *et al* (1963) have shown that the increase in myocardial contractility induced by changing afterload is associated with a net loss of myocardial potassium However the specific mechanism whereby these changes in myocardial potassium balance are induced has not been elucidated SARNOFF and associates (1963) have suggested that the changes in myocardial potassium balance induced by both frequency change and increasing aortic pressure are causally related to the associated increase in myocardial oxygen consumption However, HAJDU (1953) suggested that the loss of potassium associated with tachycardia was the result of the decrease in the interstimulus interval The experiments to be described

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were undertaken to define more clearly the relationships between myocardial performance, myocardial oxygen consumption and myocardial potassium balance

2 METHODS

Mongrel dogs of varying weights and of both sexes anesthetized with intravenously administered pentobarbital sodium (25–30 mg/kg), were used in all the experiments The preparation was an isolated blood perfused dog heart similar to that described previously (GILMORE, GERLINGS and MILLER, 1968) While maintained on positive pressure ventilation, a transverse thoracotomy was done and the heart isolated by ligating the inferior and superior vena cavae, azygos vein, lung roots, brachiocephalic artery, left subclavian artery and aortic arch immediately below the left subclavian artery The latter was then cannulated and connected to either the femoral or carotid arteries of an intact anesthetized dog (pentobarbital sodium 25–30 mg/kg) thereby providing coronary artery inflow Total coronary outflow (less left Thebesian) was diverted from the right ventricle through a low resistance rotameter and thence to a reservoir connected to the jugular veins of the support dog which breathed ambient air Left ventricular Thebesian drainage was obtained through a catheter inserted through the apical dimple Heart rate was maintained constant or varied by stimulation through electrodes sewn either to the right atrium alone or to both the right atrium and right ventricle Left ventricular performance was monitored using either an intraventricular balloon or a strain gauge arch applied to the left ventricular wall The balloon was attached to a Y shaped cannula and inserted through the apical dimple One arm of the Y served to record intraventricular pressure and the other arm was used for filling the balloon with saline The strain gauge arch was set to approximately 130 % of the resting fibre length Coronary arterial venous oxygen difference was monitored continuously using a Guyton Oxygen Analyzer [1]) which was calibrated intraexperimentally by manometric analysis Myocardial oxygen consumption was calculated as the product of total coronary venous outflow (obtained

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1) *Oxford Instrument Company, Jackson Mississippi*

by timed collection) and coronary arterial venous oxygen difference In earlier experiments coronary arterial and venous plasma potassium concentrations were determined on samples obtained by timed collection and subsequently analyzed on a Technicon Autoanalyzer In later experiments blood potassium concentration was monitored continuously using on line automated flame photometry (GERLINGS and GILMORE 1968) This technique can discern changes as small as 0 02 meq/l of whole blood potassium Changes in myocardial potassium balance were calculated as the product of either coronary arterial venous plasma potassium difference determined by planimetry and total coronary plasma flow or the product of the coronary arterial venous blood potassium difference determined by planimetry and total coronary blood flow The recordings obtained from the Autoanalyzer were digitalized by means of an Oscar Model J strip chart recorder [1]) and the numbers subsequently keyed into a remote terminal of a Burroughs B5500 digital computer A specially designed program provided conversion of the output in meq/l The program made a compensation for drift in the system assuming the drift was linear with time In the case of timed collections of arterial and venous blood the maximum recorded values of the 30 second samples were taken The continuous recording was digitalized at one measurement per 10 sec This provided a semi continuous graph since the analyzing and recording systems have a time constant of 15 sec The computer program provided output of the values in meq/l on a line printer and card punch and a graph of the K^+ levels in plasma or blood with respect to time was made on a Calcomp incremental plotter

Intraventricular tension was changed by the addition or withdrawal of saline from the intraventricular balloon Coupled pacing was done by simultaneous atrial and ventricular stimulation with paired stimuli obtained from a Grass S 4 or S 8 stimulator All drug injections were made directly into the coronary inflow line Under the conditions of the experiments to be described an increase in contractility is defined as an increase in the extent and/or rate of pressure or tension development independent of a change in initial pressure or tension All pressures were measured using Statham or Sanborn transducers The first derivative of pressure

1) Benson and Lehner Corporation Los Angeles

or tension was obtained using an R C differentiating circuit. All tracings were displayed on a Sanborn multichannel recorder.

3. RESULTS

SARNOFF *et al.* (1963) suggested that the loss of potassium associated with the adaptation of the heart to an increase in aortic pressure is the result of the concomitant increase in left ventricular developed tension. As a result of the increase in developed tension, myocardial potassium is decreased leading to an increase in contractility. To test this hypothesis, experiments were carried out in which developed tension was increased by an intervention generally believed not to change myocardial contractility, i.e., increasing preload (GILMORE and GIBBINGS, 1968). This was accomplished by adding saline to an intraventricular balloon. The hemodynamic consequences of such an intervention are shown in

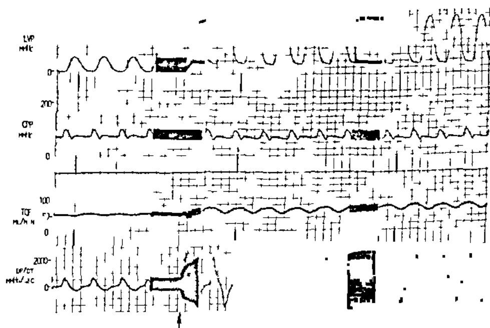


Fig. 1

The response of the left ventricle to an increase in diastolic volume. LVP = left ventricular pressure, CPP = coronary perfusion pressure, TCI = total coronary blood flow, DP/DT = the first derivative of left ventricular pressure. At the time indicated by the vertical arrow 10 ml of saline was added to the intraventricular balloon. Heart rate constant throughout. See text for further description of figure. Figure reprinted by courtesy of the Rockefeller University Press (1968).

Fig 1 (GILMORE and GEFLINGS 1968) At the time indicated by the vertical arrow 10 mL of saline was added to the intraventricular balloon Left ventricular diastolic and systolic pressure increased Associated with these changes was a small increase in coronary blood flow and a substantial increase in max dp/dt After the initial rise left ventricular diastolic pressure declined by approximately 4 mm Hg concomitantly left ventricular developed pressure increased by approximately 10 mm Hg Also while left ventricular developed pressure was increasing and left ventricular diastolic pressure decreasing there occurred a significant increase in the first derivative of left ventricular pressure Therefore, this type of experiment indicates that an increase in ventricular developed tension can be associated with an increase in left ventricular diastolic compliance (GILMORE GEFLINGS and MILLER 1968) Because of the sustained increase in left ventricular diastolic pressure in this experiment it is not possible to draw any conclusions concerning the extent to which preload might have modified myocardial

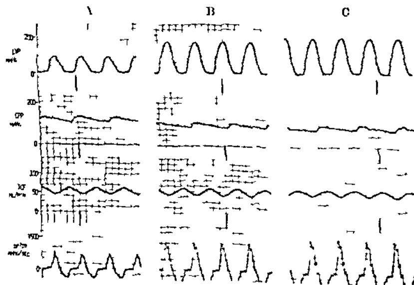


Fig 2

Fast tracings showing the influence of increasing diastolic volume on ventricular performance Abbreviations same as in Fig 1 Heart rate constant throughout Paper speed [illegible]0 millimeters per second For complete description see text Figure reprinted by permission from Circulation Research 22 763 (1968)

or tension was obtained using an R C differentiating circuit All tracings were displayed on a Sanborn multichannel recorder

3 RESULTS

SARNOFF *et al* (1963) suggested that the loss of potassium associated with the adaptation of the heart to an increase in aortic pressure is the result of the concomitant increase in left ventricular developed tension As a result of the increase in developed tension myocardial potassium is decreased leading to an increase in contractility To test this hypothesis experiments were carried out in which developed tension was increased by an intervention generally believed not to change myocardial contractility i e increasing preload (GILMORE and GIBBINGS 1968) This was accomplished by adding saline to an intraventricular balloon The hemodynamic consequences of such an intervention are shown in

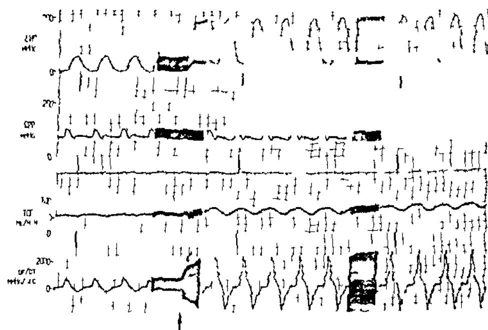


Fig. 1

The response of the left ventricle to an increase in diastolic volume LVP = left ventricular pressure CPP = coronary perfusion pressure TCI = total coronary blood flow *DP/DT* = the first derivative of left ventricular pressure At the time indicated by the vertical arrow 10 ml of saline was added to the intraventricular balloon Heart rate constant throughout See text for further description of figure Figure reprinted by courtesy of the Rockefeller University Press (1968)

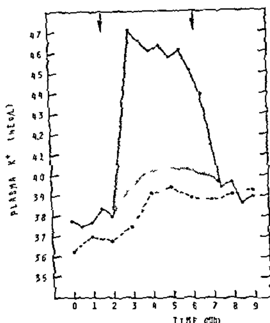


Fig 3

The influence of increasing (first vertical arrow) and subsequently *decreasing* (second vertical arrow) intraventricular volume on coronary *arterial and venous plasma* potassium concentration LVDP = left ventricular diastolic pressure Remaining abbreviations are same as in Figs 1 *and* 2 *Solid line* coronary venous plasma potassium concentration *and broken line* = coronary artery plasma potassium concentration The shaded area indicates the change in coronary venous potassium *concentration attributable* to the intervention Figure reprinted by permission from Circulation Research *22*, 769 (1968)

H R (min)	172	172
V devel P (mm Hg)	70	123
C P (mm Hg)	60	65
C F (ml/min)	66	70
dp/dt (mm Hg/sec)	1100	1500
LVDP (mm Hg)	23	62
arterial • •		
venous •——•		

contractility However, that changing preload can change myocardial contractility is *demonstrated by the type of experiment* shown in Fig 2 (GILMORE and GERLINGS 1968) Panel A is the control tracing, panel B was obtained 60 sec after increasing the volume of the intraventricular balloon with 14 ml of saline At this time diastolic pressure had returned to the pre inflation level as a result of the previously discussed change in ventricular compliance 100 sec after panel B, panel C was obtained Between panels B and C, left ventricular developed pressure and max dp/dt increased with no apparent change in left ventricular diastolic pressure In this experiment coronary perfusion pressure was allowed to decline between panels A and B and B and C in order to maintain coronary blood flow essentially constant Since as a result of the increase in developed tension produced by increasing *intraventricular volume both the extent and rate of left ventricular* pressure increased independently of a change in end diastolic pressure, it can be concluded that an increase in the preload of the heart can be associated with an increase in myocardial contractility Thus heterometric autoregulation can elicit homeometric autoregulation

It was of interest to determine to what extent the changes in performance induced by changing preload are associated with *changes in net myocardial potassium balance* An example of such an experiment is shown in Fig 3 (GILMORE and GERLINGS 1968) At the time indicated by the first vertical arrow the volume of the intraventricular balloon was increased by the addition of saline As a result left ventricular developed pressure increased from 70 to 123 mm Hg while max dp/dt increased from 1100 mm Hg/sec to 1600 mm Hg/sec These hemodynamic changes were associated with a substantial elevation of coronary venous plasma *potassium concentration* Since coronary flow increased from 66 to 70 ml/min the changes in coronary venous plasma K^+ concentration represent a net loss of myocardial potassium At the time indicated by the second *vertical arrow the volume which had been* previously added to the balloon was withdrawn Subsequently coronary venous plasma potassium concentration returned to approximately the control level Another experiment of this type is shown in Fig 4 (GILMORE and GERLINGS 1968) At the time indicated by the first vertical arrow approximately 13 ml was

added to the intraventricular balloon subsequently a substantial elevation of coronary venous plasma potassium concentration occurred At the time indicated by the second vertical arrow an additional 7 ml of fluid was added the potassium concentration of coronary venous plasma increased further Since coronary flow increased during balloon inflation these changes in coronary venous plasma potassium concentration represent net changes in myocardial potassium balance At the time indicated by the third vertical arrow the saline was withdrawn from the balloon Coronary venous plasma potassium concentration then returned close to the preinflation value Of interest in this experiment are the changes in myocardial oxygen consumption With each increase in balloon volume there occurred not only an increase in coronary venous plasma potassium concentration but also an increase in myocardial oxygen consumption These experiments demonstrate that when the tension developed by the ventricle is increased an increase in contractility can occur This increase in contractility is associated with a net loss of myocardial potassium and increase in myocardial oxygen consumption These observations raise the question as to what extent these associated phenomena are causally related However whether the loss of potassium associated with an increased developed tension is related to the change in tension or to the associated change in oxygen consumption cannot be decided from the available information

Attention was next directed towards studies designed to determine if the change in myocardial potassium balance induced by a heart rate were due primarily to the associated changes in myocardial oxygen consumption or to the associated change in the interstimulus interval Fig 5 shows the influence of changing heart rate on myocardial potassium balance (GILMORE and GERLINGS 1959) The upper tracing represents coronary arterial blood potassium concentration and the lower tracing coronary venous blood potassium concentration At the time indicated by the first vertical arrow heart rate was increased from 100 to 200 per minute At the second vertical arrow heart rate returned to 100 per minute With the onset of tachycardia a transient elevation of coronary venous blood potassium concentration occurred with cessation of tachycardia coronary venous blood potassium concentration decreased transiently No discernible change in coronary artery blood

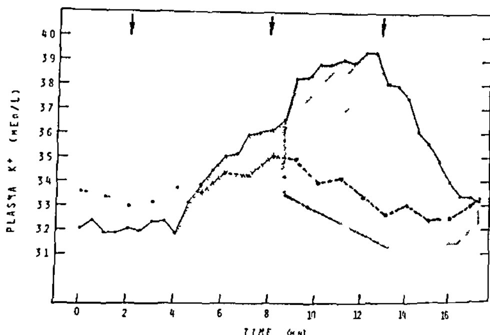


Fig 4

Influence of a step-wise increase in left ventricular diastolic volume on myocardial potassium balance. A-V O₂ difference = coronary arterial venous oxygen difference, MVO₂ = myocardial oxygen consumption; remaining abbreviations as in previous figures. The figures in the first column represent the controls; those in the second column are the values obtained after 13 ml of fluid was added to the balloon. The figures in the third column represent the values obtained after an additional 7 ml of fluid was added. The fourth column shows the new steady state values after the 20 ml of fluid was withdrawn. Figure reprinted by permission from Circulation Research 22 [illegible] (1968)

H R (/min)	130	130	130	130
V devel P (mm Hg)	75	115	153	55
C P (mm Hg)	100	90	85	60
C F (ml/min)	45	81	89	53
A–V diff (vol %)	10 5	11 6	12 8	9 8
MVO_2 (ml/min)	4 7	9 4	11 4	5 2

arterial • •
venous •——•

potassium concentration was observed Fig 6 shows the influence of changing heart rate on coronary venous blood potassium and sodium concentration (GILMORE and GERLINGS, 1969) At the time indicated by the first vertical arrow, heart rate was increased from 106 to 149 per minute At the second vertical arrow heart rate was decreased to control levels The usual transient changes in coronary venous blood potassium concentration occurred, however there was no discernible change in coronary venous sodium concentration These changes in coronary venous blood potassium concentration were usually associated with directional changes in coronary venous outflow so that these changes in concentration reflect net losses and gains of potassium by the heart Also, when heart rate was increased myocardial oxygen consumption invariably increased With the decrease in heart rate myocardial oxygen consumption returned to or towards the control levels Thus, we always found a correlation between oxygen consumption and potassium balance when heart rate was altered In order to obtain information concerning the

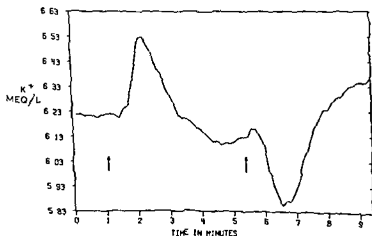


Fig 7

The influence of paired stimulation on coronary venous blood potassium concentration At the time indicated by the first vertical arrow paired pacing was initiated at an interstimulus interval of 180 msec and at the second vertical arrow paired pacing stopped Coronary artery blood potassium concentration remained essentially *constant* throughout the experiment For further description of figure see text Figure reprinted by permission from the J Applied Physiol 25, 316–318 (1968)

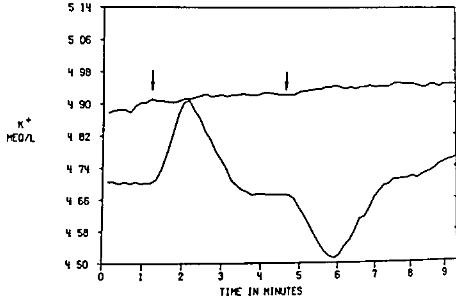


Fig 5

The influence of increasing heart rate on coronary arterial (upper tracing) and coronary venous (lower tracing) blood potassium concentration At the time indicated by the first vertical arrow heart rate was increased from 100 to 200 beats per minute and at the time indicated by the second vertical arrow heart rate was returned to 100 beats per minute See text for further discussion of figure

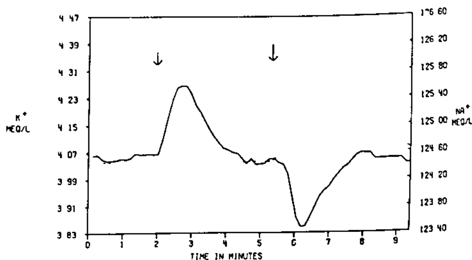


Fig 6

The influence of increasing heart rate on coronary venous blood potassium and sodium concentration The solid line represents coronary venous blood potassium and the broken line represents coronary venous sodium concentration At the time indicated by the first vertical arrow heart rate was increased from 106 to 149 beats per minute and decreased to 106 beats per minute at the time indicated by the second vertical arrow For further description of figure see text

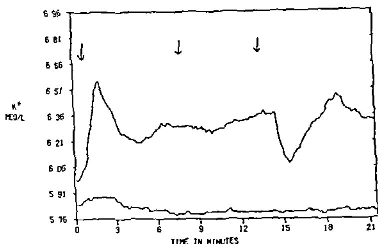


Fig 8

The influence of doubling the number of depolarizations and then doubling the number of *contractions while maintaining the number of depolarizations* constant on coronary venous blood potassium concentration The upper tracing represents coronary venous blood potassium concentration, the lower *tracing coronary* arterial *blood* potassium concentration At the time indicated by the first vertical arrow paired pacing was initiated at the time indicated by the second vertical arrow paired pacing was stopped while simultaneously doubling heart rate At the time indicated by the third vertical arrow heart rate was decreased For further description of figure see text

in myocardial oxygen consumption (1 47 ml/min) Coronary venous potassium concentration decreased transiently and then returned to essentially the level which obtained before heart rate was changed It is to be emphasized that when stimulation was changed from *paired stimulation to double* heart rate oxygen consumption declined by 2 6 ml/min but no potassium change occurred However when heart rate was subsequently decreased from 200 to 100 per minute there was a smaller decrease in oxygen consumption (1 47 ml/min) but a substantial transient net gain of myocardial *potassium Therefore these observations* demonstrate that the loss of potassium observed with increasing heart rate and paired stimulation is related not to the associated changes in *myocardial oxygen* consumption but rather to the decrease in the interval between stimuli

Although the experiments presented above demonstrate that the

relation between myocardial oxygen consumption and interstimulus interval experiments were carried out employing paired stimulation of the heart. A typical experiment showing coronary venous blood potassium concentration changes is shown in Fig. 7 (GERLINGS and GILMORE, 1968). At the time indicated by the first vertical arrow coupled pacing was instituted at an interstimulus interval of 180 m sec at a heart rate of 112 per minute. At the time indicated by the second vertical arrow paired stimulation was stopped. With the onset of paired stimulation coronary venous blood potassium concentration increased transiently; the opposite was observed when paired stimulation was stopped. During paired stimulation coronary blood flow increased from 39 to 81 ml/min and myocardial oxygen consumption increased from 10.0 to 13.4 ml/min. Although this experiment demonstrates that the increase in myocardial contractility caused by paired stimulation is associated with a net loss of myocardial potassium, this type of experiment does not provide information as to whether the loss of potassium is due to the change in the interstimulus interval or to the associated change in myocardial oxygen consumption. However, these two possibilities were distinguished by the type of experiment shown in Fig. 8 (GILMORE and GERLINGS, 1969). The upper tracing represents coronary venous blood potassium concentration and the lower tracing coronary arterial blood potassium concentration. In this experiment coronary blood flow was maintained relatively constant by altering the resistance of the coronary inflow line. At the time indicated by the first vertical arrow coupled pacing was initiated. This was associated with the usual transient net loss of potassium as indicated by the elevation of coronary venous blood potassium concentration. Coronary blood flow did not change significantly while the A-V oxygen difference increased from 8.8 to 16.1, thereby increasing myocardial oxygen consumption from 5.7 to 9.66 ml/min. At the time indicated by the second vertical arrow, coupled pacing was stopped while simultaneously doubling heart rate from 100 to 200 per minute. As a result, a slight decrease in coronary blood flow and A-V oxygen difference occurred so that myocardial oxygen consumption declined by 2.6 ml/min. However, little or no change in coronary venous potassium was observed. Heart rate was decreased from 200 to 100 per minute at the time indicated by the third vertical arrow. This was associated with a further decrease

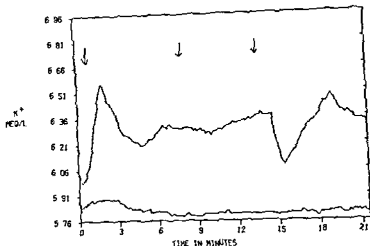


Fig 8

The influence of doubling the number of depolarizations and then doubling the number of contractions while maintaining the number of depolarizations constant on coronary venous blood potassium concentration The upper tracing represents coronary venous blood potassium concentration, the lower tracing coronary arterial blood potassium concentration At the time indicated by the first vertical arrow paired pacing was initiated, at the time indicated by the second vertical arrow paired pacing was stopped while simultaneously doubling heart rate At the time indicated by the third vertical arrow heart rate was decreased For further description of figure see text

in myocardial oxygen consumption (1 47 ml/min) Coronary venous potassium concentration decreased *transiently* and then returned to essentially the level which obtained before heart rate was changed It is to be emphasized that when stimulation was changed from paired stimulation to double heart rate, oxygen consumption declined by 2 6 ml/min but no potassium change occurred However when heart rate was subsequently decreased from 200 to 100 per minute there was a smaller decrease in oxygen consumption (1 47 ml/min) but a substantial *transient* net gain of myocardial potassium Therefore these observations demonstrate that the loss of potassium observed with increasing heart rate and paired stimulation is related not to the associated changes in myocardial oxygen consumption but rather to the decrease in the interval between stimuli

Although the experiments presented above demonstrate that the

loss of myocardial potassium associated with changing heart rate and coupled pacing is not due to the associated increase in oxygen consumption, they do not preclude the possibility that oxygen availability may contribute to changes in myocardial potassium balance when heart rate is increased. In order to investigate this possibility the type of experiment shown in Fig 9 was carried out

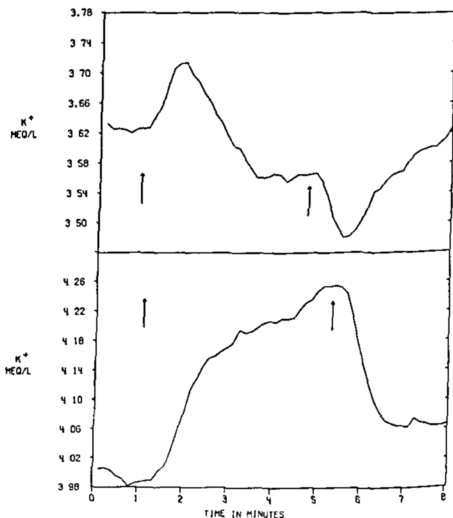


Fig 9

The influence of increasing heart rate on coronary venous blood potassium concentration before (upper panel) and after (lower panel) coronary blood flow reduction The first vertical arrow in each panel indicates the time at which heart rate was increased, the second vertical arrow in each panel indicates the time at which heart rate was subsequently decreased See text for further description of figure

(GERLINGS MILLER and GILMORE 1969) Both tracings represent coronary venous blood potassium concentration The experiment shown in the upper panel was done at normal coronary blood flow that in the lower panel was done following coronary artery inflow constriction At the first vertical arrow in the upper panel heart rate was increased from 124 to 240 beats per minute The usual transient increase in coronary venous blood potassium concentration occurred Coronary blood flow changed little (113 to 111 ml/min) while myocardial oxygen consumption increased from 8 8 to 19 4 ml/min At the second arrow heart rate was decreased from 240 to 124 beats per minute Again the usual transient decrease in coronary venous blood potassium concentration occurred Shortly after obtaining the tracing shown in the upper panel of Fig 9 coronary blood flow was reduced to 35 ml/min Heart rate was then increased from 128 to 210 beats per minute The results are shown in the lower panel of Fig 9 With the initiation of tachycardia coronary venous blood potassium concentration increased however in contrast to the experiment shown in the upper panel of Fig 9 the increase in coronary venous blood potassium concentration was maintained throughout the period of tachycardia In this experiment coronary flow changed little (35 to 33 ml/min) and myocardial oxygen consumption rose only from 5 8 to 6 2 ml/min This experiment in conjunction with those above demonstrates that although the changes in myocardial potassium balance induced by heart rate are not the result of the associated changes in myocardial oxygen consumption they do demonstrate that the extent of the K changes induced by heart rate are modified by the oxygen availability of myocardial tissue

The possible contribution of the changes in myocardial potassium balance induced by various interventions to the associated changes in myocardial performance has been discussed in detail by SARNOFF and associates (1965) and ourselves (GILMORE and GERLINGS 1968 GILMORE 1968) In general it has been shown that with hemodynamic interventions which increase contractility i e increased developed tension *tachycardia and coupled pacing* there is always an associated loss of myocardial potassium Also with drugs such as Acetylstrophanthidin the increase in contractility induced is associated with a net loss of myocardial potassium (GILMORE and GERLINGS 1968) In order to investigate this relationship further

followed by fibrillation of the heart The administration of nonactin was always associated with a substantial increase in myocardial oxygen consumption

Attention was next directed towards determining the extent to which these hemodynamic changes and changes in oxygen consumption were related to changes in myocardial potassium balance An example of one such experiment is shown in Fig 11 (GILMORE GERLINGS and MILLER, 1969) This figure shows coronary venous blood potassium concentration At the time indicated by the vertical arrow, 40 μg of nonactin were injected into the coronary artery inflow line This was associated with a substantial elevation of coronary venous blood potassium concentration Following the injection of the drug coronary blood flow increased from 56 to 100 ml/min and myocardial oxygen consumption from 7 2 to 34 2 ml/min The net loss of potassium in this experiment was 900 microequivalents Thus, with nonactin there occurs a substantial loss of myocardial potassium and an increase in myocardial oxygen consumption, however its administration is usually associated with the depression of myocardial contractility

SUMMARY AND CONCLUSIONS

The following conclusions would appear to be permissible from the experiments presented

A The extent to which tension is developed by the myocardium appears to be a determinant of its potassium balance In turn because of the associated alteration in myocardial potassium balance changing myocardial developed tension may alter myocardial contractility

B The changes in myocardial potassium balance induced by altering the frequency of contraction are the result of the change in the interstimulus interval rather than the associated change in myocardial oxygen consumption The interstimulus interval mechanism also appears to be that which influences potassium balance during paired stimulation

C Nonactin which is known to alter the permeability of cell membranes to potassium consistently produces a net loss of myocardial potassium and an associated depression of myocardial contractility

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a smaller contraction This shortened interval not only gives rise to a small beat directly following that short interval but also to a number of enlarged contractions afterwards This phenomenon is known as postextrasystolic potentiation (HOFFMAN *et al* 1956 MEIJLER *et al* 1962) The effect of one interposed longer interval is just the opposite It enhances the first contraction (rest contraction) (ROSIN and FARAH 1955) but the beats coming thereafter are somewhat smaller than the controls (depotentiation) (KATZ 1967)

With these rather simple examples it may become clear that in the intact organism in which two RR intervals are seldom identical the influence of the interval contractility relationship on the over all mechanical activity of the heart becomes rather complicated This holds for instance especially for the contractions of the heart in patients with atrial fibrillation It was found that the RR intervals of patients with atrial fibrillation are randomly distributed (MEIJLER *et al* 1968) When we knew this the effect of random rhythms on the contractile behaviour of isolated perfused rat hearts was studied By means of crosscorrelation techniques it was found that there was also a close relationship between the RR interval(s) and the height of the contraction(s) during random beating (MEIJLER *et al* 1968) These and other studies (KRUTA and BRAVENY 1968 MEIJLER and DURRER 1965) demonstrate that a direct influence of rate and rhythm on the contractile behaviour of the heart may indeed play an important role in the regulation of the circulation During every day activities the heart constantly changes its rate and rhythm and thus its RR intervals For example during exertion the heart rate increases This increase in rate enables the heart to expel more blood in a shorter time Substantial evidence has been presented that the calcium ion via the excitation contraction coupling (NIEUWENDIJK 1966 ZIMMERMAN 1966 NIEUWENDIJK *et al* 1967) translates the duration of the RR interval in a more or less forceful contraction

From the list of 9 factors I have focused attention only on factor 8 which via intermediate mechanisms influences the mechanical activity of the heart At the same time it should be realized that although the factors are listed separately they all influence each other and may even act via each other For instance the positive inotropic action of an increase in heart rate may be expressed by

a shift from one so called Starling curve to another (SARNOFF, 1955) and the effect of a rise in temperature seems to make use of an increase in heart rate (KNOWLTON and STARLING, 1912)

Once more it should be stated that the evaluation of any pharmacological intervention on the contractile behaviour of the heart is only allowable if the intervention took place during a fixed and controlled heart rate

The boundary between knowledge and ignorance of myocardial contractile behaviour has reached the subcellular level (SONNENBLICK 1968) In our country academic pharmacology is 60 years old Maybe in the coming decades the iron curtain of our ignorance will be removed from the molecular site where most probably, the contractile force of the myocardial cell is regulated The role of the heart in the regulation of the circulation may then be completely understood

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THE TRANSFER FUNCTION OF FROG MYONEURAL JUNCTIONS

I THE INFLUENCES OF FATIGUE FACILITATION, AND DEPRESSION UPON IMPULSE TRANSMISSION *IN VITRO*

BY

A. C BOBBERT

CONTENTS

1. INTRODUCTION, PLAN OF INVESTIGATION

In the familiar classroom experiment where "contraction summation" is studied with indirect stimulation of the frog's sciatic-gastrocnemius preparation it may be observed that there are wide differences in behaviour between preparations, though in all cases single stimulus pulses are supramaximal, the two pulses of the double stimuli are equal to the single pulses, and the pulse interval in the two-pulse stimuli is the same in all cases. For a given pulse interval the ratio of the height of the contraction elicited by a twin pulse and that caused by a single pulse varies widely between preparations.

This difference cannot readily be explained on the basis of what is known about contraction summation in the case of direct stimulation. Since, in addition to this, it appeared that the behaviour of the preparation depends in part on its history, *i.e.* on the number of stimuli applied to the nerve before the "summation" experiment proper, it seemed useful to study this matter further.

2. MATERIALS AND METHODS

2.1. Basic procedures

In all experiments the preparations consisted of the sciatic nerve and gastrocnemius muscle of *Rana Temporaria* or *R. Esculenta* of either sex and of approximately the same size. They were usually examined *in vitro* in a bath of Nastuk-solution or paraffin-oil; only in a few cases the preparation was left *in situ*, in decerebrate animals in which the nerve was sectioned and freed from the surrounding tissues without causing appreciable blood loss or obstruction of the circulation through the muscle, after which the circulation through the other muscles was tied off and their motor nerves were sectioned.

Only fresh preparations were used in the experiments, which were performed, in all seasons, in a shielded room in which the temperature was kept at 18–21° C. It appeared that the results

were not appreciably influenced by the season or by the animal's sex

2 2 *Stimuli* were applied to the nerve by means of platinum electrodes, or directly to the muscle by means of steel electrodes clipped on to the tendo calcanei and to the femur The pulses were delivered by a square wave generator triggered by a multiple time-delay generator providing triggers at the rate of 1 per 10 sec Any of these single pulses could be replaced by a double pulse with an adjustable interval between the members of the twin or by a train of 3 to 5 pulses with preset intervals

2 3 *Recording* was done by mechanical and electrical means *Mechanical records* were obtained with a conventional isotonic lever, writing on a long-extension kymograph *Electrical records* were obtained either from a macro electrode placed close to the belly of the muscle or piercing its tendon and providing the overall-electromyogram, or from a steel micro-electrode, with a bare tip length of 3–15 μm, which signalled action potentials, and sometimes endplate potentials, in single muscle fibres From both types of electrode the potential changes were led off against an earthed electrode and fed into the input stage of a Tektronix pre amplifier type 122, mod 151 The output of this pre amplifier, the gain of which was set at 1000× while the variable low pass filter was set at an R-C time of 2 msec, was connected with the input of a Tektronix 502A oscilloscope, from the screen of which single shot photographs were taken at preset intervals Besides this, the responses were made audible by means of an audio amplifier and a loudspeaker

2 4 *Sequence of events* during a typical experiment Pulses of 1 msec duration were used throughout In most experiments they were of supramaximal intensity Prior to the experiment proper, 1 msec test pulses of stepwise increasing voltage were applied to determine the "maximal" stimulus voltage, the number of these test pulses never exceeded seven In the experiments themselves, stimulus strength was at least 115 pct of the just maximal pulse intensity, a stimulus was applied every ten seconds, five out of six stimuli consisted of single supramaximal pulses, every sixth stimulus was either a 25 msec twin or a train of 3 to 5 equal pulses.

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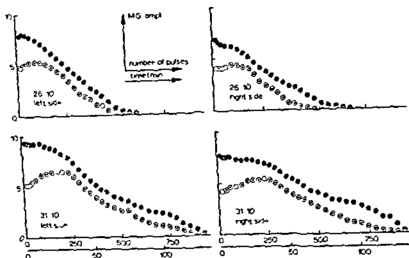


Fig 1

Changes in amplitude of the mechanical responses of 4 muscles to directly applied supramaximal single pulses (○) and 25 msec twins (●)

msec twin is nearly twice as large as that elicited by application of a single pulse During the next 18 min in the muscles of the one frog, and for 30 min in those of the other, the contractions evoked by single pulses increase markedly in amplitude while those elicited by twin pulses diminish slightly After that, both decrease according to nearly parallel curves It further appears that, while intra frog differences in the time course of M G amplitude changes are slight, there are marked inter-frog differences

To reduce the effect of inter-frog differences the mechanograms of 4 directly stimulated muscles were averaged The results are shown in the upper part of Fig 2 (left), together with those obtained in the same way from the preparations in which the muscles were stimulated indirectly (right) In the lower part of this figure the changes in resting length of the muscles are plotted at the same scale It appears that the resting length increases rapidly during the first 30–50 min of stimulation

If in Fig 2, the curves for the changes in the mechanical response to single pulses for direct (left) and for indirect stimulation (right) are compared, it becomes evident that in the latter case the initial increase of the responses to single pulses (staircase effect,

The electrical responses to these double pulses or pulse trains were photographed at preset intervals In these records the response due to the first pulse obviously corresponds with the response to a single pulse applied at that moment The amplitudes of the electrical responses to the stimuli were read off in millivolts, those of the mechanical responses to a twin pulse or a pulse train and to the immediately preceding single pulse were expressed in arbitrary units Unless indicated otherwise the pulse interval of the twins was 25 msec

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3 RESULTS OF STIMULATION WITH SUPRA-MAXIMAL PULSES

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3 1 1 *Mechanograms during direct and indirect stimulation*

Experiments with *direct* stimulation were made on 4 muscles taken from two decerebrate frogs 30 min after an intra abdominal injection of 80–100 mg Flaxédil

The experiments were made according to the standard scheme for stimulation in which a double pulse of 25 msec interval re placed every sixth single pulse during stimulation at the rate of 1/10 sec Because the stimulus artifacts which result from the high voltage pulses needed for direct stimulation of all muscle fibres largely obscured the electromyograms (EMG s) only the mechanograms (MG s) could be used In another 4 preparations stimuli were applied *indirectly* according to the same scheme In these experiments the amplitude of the artifacts was small com pared to that of the electrical responses of the muscles so that both the mechanical and the electrical records could be used

Fig 1 shows for each of the 4 directly stimulated muscles how the mechanical responses to single pulses and to twins change in the course of 1 per 10 sec stimulation during 120 min

At the start of stimulation the shortening in response to a 25

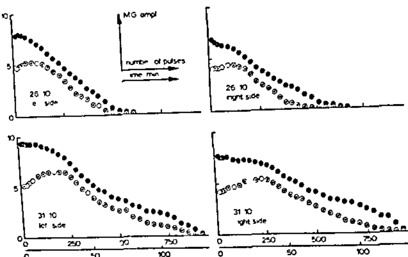


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This is more clearly apparent from Fig 3 in which the ratio between the degrees of shortening due to double and to single pulses has been plotted against the number of preceding stimuli, for both types of activation In both cases the ratio is the same at the onset of stimulation, it then shows a transient decrease which is more marked and lasts longer in the case of direct stimu-

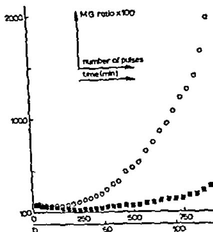


Fig 3

Same preparations as in Fig 2 Changes in the ratio between the amplitudes of the mechanical responses to double and to single pulses in the course of direct (■) and indirect (O) stimulation.

lation, the ratio then increases slowly during direct stimulation and steeply in the case of indirect activation This marked increase of the ratio during indirect stimulation indicates that the behaviour of preparations activated in this way is indeed largely dependent on the number of previously applied stimuli (*cf* section 1)

From Fig 3 it becomes evident that the wide scattering of this ratio in classroom experiments is due to at least 3 phenomena, *viz*

- the staircase phenomenon that is present in the responses to single pulses and nearly absent in those to 25 msec double pulses, and which lowers the ratio during the staircase period As it is present both with indirect and with direct stimulation it must be due to a property of the muscle fibres It was earlier observed during *in vitro* experiments

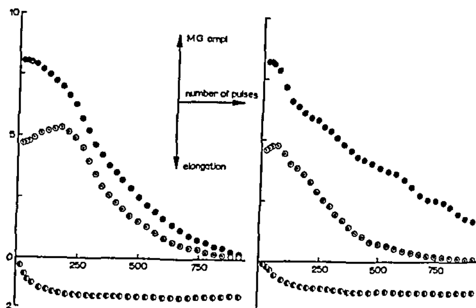


Fig 2

Changes in resting length (◐) and in average amplitude of the mechanical responses to single (○) and to 25 msec double pulses (●) in the course of direct (left) and indirect (right) stimulation

"Treppe") is less pronounced than in the case of direct stimulation, and that it lasts for a much shorter period

In the case of *direct* stimulation, during the staircase period, the contractile response increases with increase of resting length, which suggests that the staircase phenomenon results from the elongation of the muscle fibres, in accordance with Starling's Law In the *indirectly* stimulated muscles, however, the staircase period is much shorter than that in which the resting length of the muscle fibres increases This suggests that during activation by way of the nerve another factor comes into play which shortens the stair case period

After the staircase period the amplitude of the contractions due to single pulses declines in a fairly similar way for both types of activation If, on the other hand, the mechanical responses to double pulses are compared for the two types of activation, it appears that, after an initial period of some 35 min in which these responses are nearly identical, the amplitude of the contractions due to indirectly applied double pulses declines at a much slower pace than in the case of direct stimulation

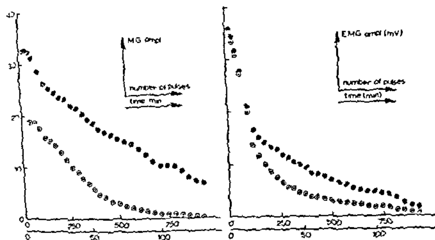


Fig 4
Changes in average amplitudes of the mechanical (left) and electrical (right) responses to indirectly applied single pulses (○) and 25 msec twins (●)

the amplitudes of the mechanical responses to double and to single pulses begins its rapid rise in the case of indirect stimulation (Fig 3)

Because these are extracellular records picked up far from the neuromuscular junctions the contribution of the endplate potentials to these responses must be negligible (DEL CASTILLO and KATZ 1956) and these response changes must therefore reflect changes in the overall response of the electrically excitable region of the muscle fibre membranes Theoretically such changes might result from

- desynchronization of the action potentials of individual muscle fibres,
- decrease in amplitude of these potentials,
- decrease in the number of muscle fibres responding to the stimulus with a propagated spike This might be due to a decrease in the response/stimulus ratio of muscle fibres, i e that for a given fibre there is a decrease of the probability that it fires upon the arrival of an impulse at its junction If this is really the case then the small difference between the responses to the first and second pulses at the onset of stimulation and the large increase of this difference later

on muscles of rats (PAUL, 1961), frogs (MASHIMA *et al.*, 1962) *and toads* (ALJURI and BORRERO, 1968), and has been attributed to an accumulation of potassium ions in the interstitial spaces and to a strengthening of the excitation contraction coupling, both supposed to result from repeated activation during anoxia. While the possible occurrence of such changes cannot be denied, it seems more probable that the staircase effect results from changes in the resting length of the muscle fibres, especially because it even appears in ageing preparations which are *not* repetitively stimulated (cf. 3.2.1, Fig. 11).

- the rather slight, and gradual increase of the ratio which *occurs in the case of direct* stimulation after the staircase period. It is highly probable that this results largely or wholly from the inertia of the recording system which exaggerates the differences in contraction height.
- the very steep increase of the ratio between the degrees of shortening that occurs, in the case of *indirect* stimulation, after the staircase period. This phenomenon must be related to synaptic and/or presynaptic processes, a conclusion which is strongly supported by the behaviour of the electrical responses to the 25 msec double pulses during indirect stimulation (3.1.2.).

3.1.2. *Electromyograms during indirect stimulation*

The electrical overall responses of the muscles to the first and to the second pulses of 25 msec twins in the case of supramaximal indirect stimulation, are plotted against the number of stimuli administered in Fig. 4 (right) together with the mechanical responses to single and to double pulses (left, *cf.* Fig. 2). It appears from this figure that the amplitude of the electrical responses to both pulses of a 25 msec twin diminishes when the number of applied stimuli increases and, further, that the amplitude of the response to the second pulse is larger than that evoked by the first *from the start of stimulation*, although both pulses are supramaximal and must each initiate an impulse in every fibre of the nerve. It further appears that the ratio between the amplitudes of the electrical responses to the second and to the first pulses increases markedly after the staircase period, i.e. at the time when the ratio between

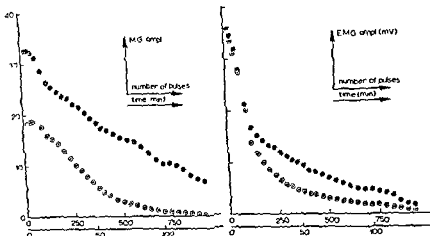


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on might be explained by assuming that the first pulse of each twin has a facilitating effect on the response of the muscle fibres to the second, *i e* that for the second pulse the response/stimulus ratio is enhanced The correctness of the two assumptions invoked was confirmed when records were made of the action potentials of individual muscle fibres during similar experiments

3 1 3 The *response/stimulus ratio* for the case of individual muscle fibres was determined in preparations indirectly stimulated according to the standard scheme (2 4)

After the muscle had been fixed in a tautly stretched condition, in order to minimize movements of its fibres a micro electrode was placed with its tip on the muscle surface where it picked up the action potentials from a single muscle fibre The stability of the recording was checked auditively as well as visually

Although units were frequently lost during prolonged stimulation, records of long duration were obtained from a few For these the response/stimulus ratio was determined every minute by counting the total number of spikes elicited by the five single pulses and by the first pulse of the twin applied every minute, and dividing this number by 6

In Fig 5 the ratio has been plotted against time and against the number of preceding stimuli, for 3 muscle fibres If these 3 fibres may be considered as a more or less representative sample from the whole population, it can be stated that at the onset of stimulation the response/stimulus ratio is indeed lower than 1 1 for part of the neuromuscular junctions and that it decreases rapidly during indirect stimulation

During extracellular recording from single muscle fibres it was repeatedly observed that the amplitude of the action potentials decreased somewhat before the spike responses disappeared completely The largest decrease observed amounted to only 20 pct of the initial spike amplitude, in agreement with observations by MASHIMA *et al* (1962) and ORKAND (1963)

From the behaviour of the response/stimulus ratio of the fibres shown in Fig 5 it may be concluded that at the onset of stimulation *in vitro* not every muscle fibre always responds to a supramaximal single pulse applied to the nerve with the firing of an action potential During continued 1 per 10 sec stimulation the

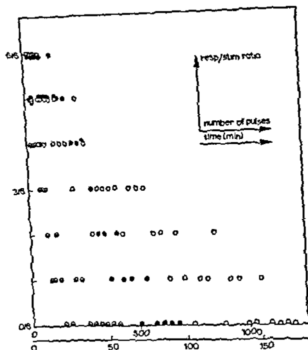


Fig 5

Response/stimulus ratio of 3 muscle fibres (A ○, B ● and C ◒) during 1 per 10 sec stimulation of the motor nerve

number of responding fibres decreases rapidly with the result that after prolonged stimulation the firing level is attained in a few muscle fibres only, while the majority have dropped out (compare the units A and C in Fig 5) So the first assumption, according to which the decline in the electrical responses to single pulses (Fig 4) results mainly from a progressive decrease of the response/stimulus ratio of the muscle fibres, proved to be correct, while the decrease in amplitude of the action potentials serves as a contributing cause

The experiments just described do not allow of a conclusion as to the possible contribution of desynchronization

It was further observed that the response/stimulus ratio of muscle fibres, after having been reduced to zero by prolonged stimulation via the nerve, was not temporarily increased after interruption of the stimulation This is in agreement with an earlier observation according to which there is no

appreciable recovery of the overall responses in the experiments referred to in section 3 1 2

In these *single unit experiments the second assumption* proved to be valid too because it was repeatedly observed that, if a muscle fibre fires in response to the first pulse of a 25 msec twin it nearly always fires again in response to the second, and further that, if the synaptic transmission ratio in the case of single pulses has been strongly reduced by prolonged stimulation, it is often seen that the fibre fires in response to the second pulses of the twins although it fails to respond to the first ones An example is given in Fig 6, in which each record shows the superimposed responses of a muscle fibre to the 10 double pulses applied during 10 consecutive minutes At the start this fibre responded with a spike to 8 out of 10 single pulses (Figs 6a and 6b) while it fired in a 1 1 fashion in response to the second pulses of 10 twins (Fig 6b)

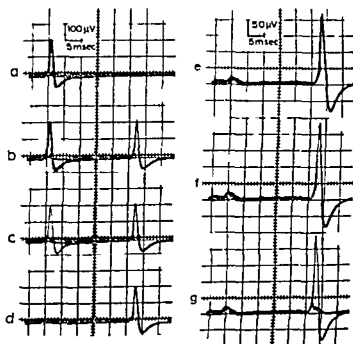


Fig 6

Electrical responses of a single muscle fibre to indirect stimulation Each picture shows 10 superimposed records of the response to a single pulse (a) or to a 25 msec twin (b g) For a better recording of the EPP s the records in e g were made at twice the amplification used in a d

Later it responded only to 2 of the 10 first pulses, while it still fired 1 1 in response to the second pulses (Fig 6c) Still later the spike responses to the first pulses disappeared completely although responses to the second pulses occurred every time (Fig 6d, e, and f), until finally these too disappeared (Fig 6g) In this case the electrode happened to be situated in the vicinity of the motor endplate with the result that, if a given pulse did not give rise to a spike, there appeared an endplate potential which was not distorted by an action potential

From Fig 6g, in which the EPP s following the first and second pulses of the twins are to be seen, it appears that the EPP's elicited by the second pulses are larger than those evoked by the con ditioning first pulses This is more evident in the upper record of Fig 7, which was obtained from another muscle fibre and where

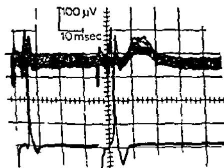


Fig 7
Superimposed records of the electrical responses of a muscle fibre (upper part) and of the whole muscle (lower part) to 10 indirectly applied 30 msec twin pulses

the EPP s due to the second pulses of the (30 msec) twins stand out clearly, while those due to the first pulses are so small as to be drowned in the accompanying action potentials It may there fore be concluded that the first pulse of a twin has a conditioning action reflected by the occurrence of a larger EPP and a markedly raised response/stimulus ratio, which lasts for at least 30 msec

Because these marked after effects appear to have a time course differing from that of unfacilitated EPP's in twitch fibres of the frog at room temperatures (FATT and KATZ, 1951), it would be

interesting to know their full time course But it is obvious before hand that the magnitude and duration of such a conditioning effect, as measured by the increase of the response/stimulus ratio for the second pulses of twins, will be influenced by the degree to which the response/stimulus ratio for the first pulses was lowered by the preceding stimuli

3 1 4 *Time course of the facilitatory after-effect of a single pulse*

The time course of the facilitatory effect of a single pulse on the transmission ratio for a closely following test pulse was de termined in experiments in which the interval between the pulses of the twins was varied Apart from this, stimulation followed the standard pattern The interval between the members of a twin was changed every minute according to a scheme in which a series of intervals was presented first in the order from the longest value to the shortest and then in the reverse sequence In this way a double pulse with a given interval was applied twice during each series and the results of these *in duplo* measurements were averaged in order to minimize the systematic errors arising from changes in the preparations in the course of stimulation *in vitro* In each experiment the standard series of twin pulses with varying interval was applied thrice, during successive periods of about 35 min Experiments were made both with indirectly and directly applied pulses of supramaximal strength From 4 preparations the electro myograms and the contractions were recorded during indirect stimulation The results are represented in Figs 8 and 10

From Fig 8 it appears that the ratio between the amplitudes of the EMG's following the second and first pulses of a twin depends, for a given interval, on the number of preceding pulses At the start of the experiments the second EMG is somewhat smaller than the first over nearly the whole range of intervals, except those between 8 and 50 msec (graph A) Later the ratio increases rapidly, as is apparent from a comparison of graphs B and A, and the EMG following the second pulse becomes the larger one for the whole range of intervals from 5 to at least 175 msec (graph B) After application of some 450 pulses the ratio does not change significantly any more (compare graphs C and B) although the EMG amplitudes continue to decrease for both pulses It further appears that the ratio between the amplitudes of the two EMG's

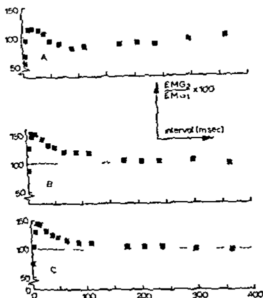


Fig 8

Indirect stimulation Ratio between the average amplitudes of the electrical responses to the second and to the first pulses of supramaximal twins plotted against the interval between the members of the twins For the meaning of A B, and C see text.

depends strongly on the interval between the pulses of a twin, it has a peak value in excess of 100 pct at an interval of some 17 msec after the conditioning pulse, which is followed, in the earlier part of the stimulation period, by a temporary decrease below 100 pct (graph A) In the case of continued stimulation this decrease is absent and there is only a phase of facilitation which decays gradually from its peak value until it ends after some 300 msec (graphs B and C)

From single unit records (Fig 6) it appeared that such a difference between the amplitudes of the two EMG's due to a twin pulse results from the fact that in numerous muscle fibres the response/stimulus ratio for the second pulse is raised above that for the first This conclusion is further supported by the observation that the rise in response/stimulus ratio of many muscle fibres has a time course similar to that of the EMG An example of this is

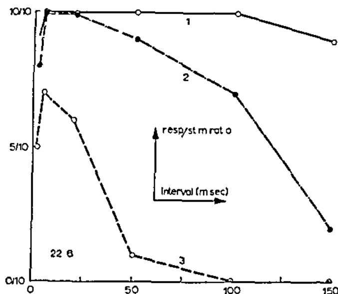


Fig 9

Response/stimulus ratio of a muscle fibre for the second pulses of twins plotted against the interval between the twin members for 3 successive periods of indirect stimulation marked 1 2 and 3 respectively

presented in Fig 9 in which for a single muscle fibre the relation is shown between its response/stimulus ratio for the second pulses of twins and the interval between these and the conditioning first pulses The curves 1–3 were obtained in successive periods of stimulation according to the standard pattern

This fibre fired in response to the first pulses of the twins only during the period in which the data presented in curve 1 were collected

From Fig 9 it is evident that a conditioning pulse has an after effect capable of raising the transfer ratio for a second pulse over a period of more than 150 msec (curve 1) and further that for this fibre the conditioning effect has a peak value after some 5–20 msec These observations together with similar ones on the *in vitro* behaviour of other units explain why the difference between the responses of a nerve muscle preparation to the second and first pulses of twins follows a similar time course (Fig 8)

This time course of the facilitatory effect of an impulse resulting from a conditioning stimulus on the response/stimulus ratio is closely similar to that of facilitation (primary potentiation) as measured by the increase in amplitude of the EPP after a con

ditioning stimulus (FENG, 1940, 1941, LUNDBERG and QUILISH, 1953a, b, DEL CASTILLO and KATZ 1954c, HUBBARD, 1963, HUBBARD and SCHMIDT, 1963, THIES, 1965, KATZ and MILEDI, 1966, 1968, MALLART and MARTIN, 1967, RAHAMIMOFF, 1968)

For the experiments with *indirect* stimulation (in which the ratio between the EMG amplitudes changes according to the curves of Fig 8) the changes in the ratio between the mechanical response to a double pulse with a given interval and that to the immediately preceding single pulse are plotted against the intervals in Fig 10, together with comparable results obtained with *direct* stimulation of another 4 muscles The curves for the ratio of the mechanogram amplitudes during indirect stimulation are similar to those for the EMG amplitudes save that here the response to double pulses is larger than that to single pulses for the whole range of intervals from 2 up to over 200 msec (compare the curves A in Figs 10

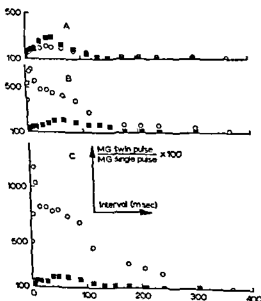


Fig 10

Ratio between the average amplitudes of the isotonic contractions in response to twins and to single pulses plotted against the interval between the members of twins, for directly (■) and indire Octly () applied stimuli Curves A B and C were obtained in periods corable with thmpaose of Fig 8

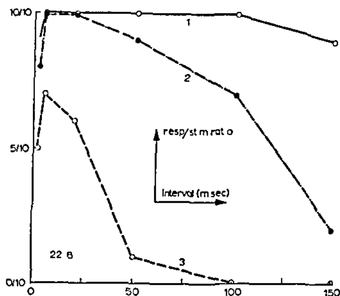


Fig 9

Response/stimul is ratio of a muscle fibre for the second pulses of twins plotte l against the interval between the twin members for 3 successive periods of indirect stimulation marked 1 2 and 3 respectively

presented in Fig 9 in which for a single muscle fibre the relation is shown between its response/stimulus ratio for the second pulses of twins and the interval between these and the conditioning first pulses The curves 1–3 were obtained in successive periods of stimulation according to the standard pattern

This fibre fired in response to the first pulses of the twins only during the period in which the data pr sented in curve 1 were collected

From Fig 9 it is evident that a conditioning pulse has an after effect capable of raising the transfer ratio for a second pulse over a period of more than 150 msec (curve 1) and further that for this fibre the conditioning effect has a peak value after some 5–20 msec These observations together with similar ones on the *in vitro* behaviour of other units explain why the difference between the responses of a nerve muscle preparation to the second and first pulses of twins follows a similar time course (Fig 8)

This time course of the facilitatory effect of an impulse resulting from a conditioning stimulus, on the response/stimulus ratio is closely similar to that of facilitation (primary potentiation) as measured by the increase in amplitude of the EPP after a con

interval for contraction summation remains at some 50–60 msec

It is now possible to explain the differences between preparations, observed during classroom experiments in which contraction summation is studied with indirect stimulation of frog muscles (1)

These differences clearly arise from the previous history of the preparations, i e from the number of pulses applied and from the passage of time By far the most important factor seems to be the change in response/stimulus ratio of individual muscle fibres (3 1 2 , 3 1 3) If only a few stimuli have previously been applied, the response/stimulus ratio for supramaximal single pulses is 1 1 for the majority of fibres and the second pulse of a twin, with the same voltage as the first, cannot "recruit" more than a few muscle fibres in addition to those which already responded to the first pulse As a result, the difference between the contractions due to double and to single pulses is then mainly caused by "pure" contraction summation (*cf* Figs 1–3 and 10) But, during repeated stimulation there occurs for all muscle fibres a progressive lowering of the response/stimulus ratio for single pulses and, thus, also for the first pulses of 25 msec twins (Figs 5 and 6), while for the second pulses of such twins the response/stimulus ratio is heightened in numerous fibres by the facilitating after effect of the first ones (Figs 6 and 9) This conditioning effect of a pulse depends upon a synaptic or pre synaptic phenomenon which follows the time course depicted in Figs 8 and 9 and causes, after the application of some 100 pulses *via* the nerve an increasing divergence between the electromyograms following the second and first pulses of 25 msec twins (Fig 4) and between the corresponding mechanograms (Fig 3)

Thus, it may be concluded that during stimulation *via* the nerve "contraction summation" stems largely from a post-synaptic phenomenon in "fresh' preparations to which only a few pulses have been applied but that, after repetitive stimulation, the difference between the mechanical responses depends upon both contraction summation and recruitment The contribution of recruitment apparently increases strongly in the course of prolonged *in vitro* experiments with a very low rate of stimulus repetition

The results of the experiments described above naturally raise the question whether the observed decreases of the mechanical and electrical responses in the course of these long lasting experi-

and 8) Again, the ratio has a maximum that increases in value during continued stimulation, but for this ratio the time course changes during stimulation because the maximum shifts from an initial interval value of some 40 msec to one of about 7 msec (compare the graphs A to C of Fig 10)

This unexpected change in the time course of the MG ratio during continued *indirect* stimulation led us to repeat these experiments, but now with *direct* stimulation

3 1 5 *Time course of summation of contractions during direct stimulation*

This has already been plotted in Fig 10, together with that for indirect stimulation The results shown in graphs A C were obtained in corresponding periods for both types of stimulation From Fig 10 it appears that, for twin pulses with a given interval, the change in the MG ratio during direct stimulation is far less than that during stimulation *via* the nerve It further appears that in the case of direct stimulation the differences between the mechanical responses to double and to single pulses are negligible for twins with intervals of 200 msec and more, but that, upon shortening of the interval, they increase towards a maximum at an interval between 50 and 60 msec, which shows, in the course of repetitive stimulation, *no* tendency to shift towards shorter intervals

This might have been predicted because, with *direct* stimulation, any difference between the responses to a twin and to a single pulse can arise only from pure contraction summation Therefore it must follow the time course of a single twitch which indeed lasts for some 150–200 msec and has its peak at about 55 msec

These results indicate that in the case of *indirect* stimulation the shift of the maximum must arise from a synaptic or presynaptic phenomenon, which clearly plays a rôle from the onset of stimulation because, already then, the ratio is maximal at an interval that is 15 msec shorter than the optimal interval for contraction summation During continued application of indirect stimuli the maximum for the amplitude ratio of the contractile responses shifts towards the same interval of some 7–10 msec where the ratio between the amplitudes of the electrical responses has its peak value (compare Figs 10 and 8), while the optimum

together with the similarly expressed changes that occurred during direct stimulation at the rate of 1 per 10 sec (3 1 1) From the ageing experiments it appears that, after the staircase period which is, again, more marked for the response to single pulses than for that to twins, the mechanical responses to both decrease according to nearly the same curve After 120 min of deterioration the mechanical responses to single pulses and to 25 msec twins are still at about 70 pct of their initial values while in the stimulation experiments, after the same period, they are down to 10 pct for single pulses and to 22 pct for twins This difference is obviously due to the difference in the number of applied stimuli This post-synaptic fatigue might be caused by

- a decrease in electrical excitability of the muscle fibre membranes due to hyperpolarization This is highly improbable, quite apart from the observation that the action potentials *decrease* in amplitude (3 1 3)

  As to this the reports in the literature are conflicting some stating that the electrical excitability decreases markedly (GERARD and JENERICK, 1953, PAUL, 1961) while according to others it does not change appreciably (RUSHTON 1933, BROOKS and THIES 1962)

- a weakening of the excitation contraction coupling due to an increasing shortage of the energy needed for the removal of freed calcium ions from the sarcoplasmatic reticulum and for re-storing them at the inside of the muscle fibre membranes
- a decrease in the contractility of the actin myosin filaments, due to an increasing shortage of the energy needed for contraction and to the accumulation of metabolites during anaerobic contractions (DEL POZO, 1942)

Fig 12 shows the normalized changes in the mechanical responses to *indirectly* applied single pulses (left) and twins (right) during ageing and in the course of the earlier experiments with indirect stimulation at the rate of 1 per 10 sec (3 1 1) In the ageing preparations there is, during the first 120 min, *no* difference between the decrease of the mechanical responses to test stimuli applied indirectly (Fig 12) or directly (Fig 11) Afterwards the

ments arise from changes due to the increase in number of the stimuli applied or to a deterioration of the preparations with time. Probably, they result from both factors and it would be useful therefore, to know the relative magnitudes of the contributions from "fatigue" and from "ageing" of the preparations to the aforementioned results. For this reason the influence of ageing was studied separately.

3.2 CHANGES IN RESPONSES DUE TO AGEING OF *in vitro* PREPARATIONS

The influence of ageing of preparations on their responses to stimulation according to the standard pattern (2.4.) was investigated in experiments lasting for 4 h (*i.e.* twice the duration of the previous experiments), while stimulation was restricted to test stimuli applied at intervals of 30 min. These test stimuli, applied directly to 4 muscles and indirectly to another 4, consisted of a supramaximal single pulse followed after 10 sec by a 25 msec double pulse of equal voltage. The results of these experiments are expressed in Figs. 11–13 as the ratio between the actual amplitude of a response and its initial value.

3.2.1 *Changes in mechanograms due to ageing*

In Fig. 11 the normalized changes in amplitude of the mechanograms in response to *directly* applied single pulses (left) and 25 msec twins (right) during ageing of the preparations are given.

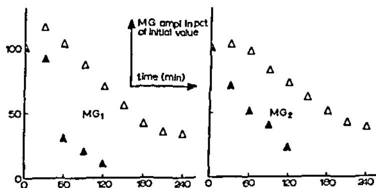


Fig. 11

Normalized changes in the average amplitude of the mechanical responses to directly applied single pulses (left) and 25 msec twins (right) caused by 1 per 10 sec stimulation (▲) and by ageing (△).

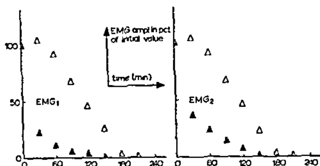


Fig 13

Normalized changes in the average amplitude of the electrical responses to the first (left) and to the second (right) pulses of indirectly applied twins, in the course of 1 per 10 sec stimulation (▲) and as a result of ageing of the preparations (Δ)

according to the same curve, there is a rapid dissociation between the curves for the second and first pulses of the twins This dissociation was mentioned before (3 1 2) and was found to depend on a conditioning effect of the first pulse upon the response/stimulus ratio for the second (3 1 4), a facilitation which follows the time course shown in Fig 9 From Fig 14, in which the ratio between

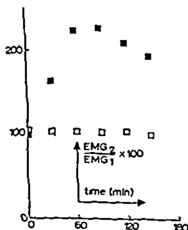


Fig 14

Ratio between the average amplitudes of the electrical responses to the second and to the first pulses of indirectly applied 25 msec twins for ageing preparations (□) and for muscles stimulated at 1 per 10 sec (■)

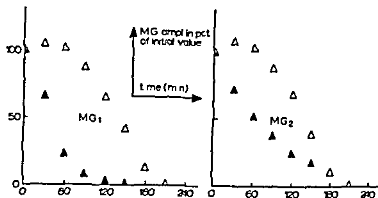


Fig 12
The same as in Fig 11, but now for indirect stimulation

between the decrease in amplitude of the mechanical responses during stimulation and that caused by ageing alone represents the fatigue due to the repetitively applied stimuli If this fatigue is compared with that during direct stimulation (Fig 11), it appears that the decline of the mechanical responses to indirectly applied single pulses, though largely due to postsynaptic changes, also has a synaptic or presynaptic component This suggestion is strongly supported by the conclusions that can be drawn from the simultaneously occurring changes in amplitude of the electromyograms (3 2 2)

3 2 2 *Changes in electromyograms due to ageing*

Fig 13 pictures the normalized changes in amplitude of the EMG's following the first (left) and second pulses (right) of indirectly applied 25 msec twins of supramaximal voltage both for the ageing preparations and for the previously described (3 1 2) experiments with 1 per 10 sec stimulation It is obvious that in the ageing experiments after a period in which the electrical responses to the first and second pulses of a twin are slightly increased, both amplitudes diminish according to the same curve If this curve is compared with that of the effect of ageing on the mechanical responses (Fig 12) it appears that the rate of decline of the electrical responses hardly exceeds that of the contraction amplitudes Fig 13 further shows that during 1 per 10 sec stimulation the electrical responses decrease far more rapidly than during ageing alone, but it also appears that *these* responses do *not* decline

On the other hand, it was concluded from the experiments with repetitive indirect stimulation (3 1) that the decrease in the mechanical and electrical responses results partly from a synaptic change which increases with the number of applied stimuli Reports on the occurrence of a decrease in acetylcholine sensitivity of the subsynaptic membranes in similar circumstances are highly conflicting (KATZ and THESLEFF, 1957, KRNJEVIC and MILEDI, 1958a b, 1959 THESLEFF, 1959, OTSUKA *et al*, 1962) It seems probable therefore, that the synaptic component of the fatigue which occurs in frog muscles during indirect stimulation *in vitro* is largely due to a progressive decline in quantum content of the EPP s This too has previously been observed at the neuromuscular junctions of frogs (DEL CASTILLO and KATZ, 1954c, OTSUKA *et al*, 1962) and toads (FENG, 1941), and at the motor endplates of various mammals, including man (LILEY and NORTH, 1953, THESLEFF, 1959, STRAUGHAN, 1960, KRNJEVIĆ and MITCHELL 1961, BROOKS and THIES, 1962, OTSUKA *et al*, 1962, HUBBARD, 1963, BEANI *et al*, 1964, ELMQVIST and QUASTEL 1965, THIES, 1965)

A similar decrease in the number of transmitter quanta released per impulse appears, during stimulation at higher frequencies at the endings of Ia afferent fibres of the cat (EVANSON, 1956, LLOYD and WILSON, 1957, CURTIS and ECCLES 1960), in the preganglionic fibres of cats (PERRY, 1953, BIRKS and MACINTOSH 1957, 1961, MACINTOSH 1959) and toads (NISHI *et al*., 1967), and at the adrenergic endings of fibres innervating the cat s spleen (BROWN *et al*, 1961 DAVIES, 1963)

The decline in number of the quanta released per impulse at frog motor endings might result from a gradual decrease in amplitude of the presynaptic action potentials (HUBBARD, 1963, HUBBARD and LØYNING 1966, MARUHASHI and WRIGHT, 1967) It may also be that the decrease of the responses to indirectly applied pulses present both in the ageing experiments and during stimulation (Figs 12 and 13), is caused to a larger or lesser degree, by a decrease of the electrical excitability of the nerve fibres In order to evaluate the possible contribution from such presynaptic changes to the results of the present investigation recordings were made from nerves themselves

3 2 3 *Changes in electroneurograms*

Fig 15a shows, for 4 ageing nerves, the normalized changes which

the EMG's due to the second and first pulses is plotted for the ageing experiments and for those with 1 per 10 sec stimulation, it may be concluded that a similar increase of the response/stimulus ratio for the second pulses does not occur during the first 3 h of ageing alone, *although in this case too there is a marked decrease in amplitude of both EMG's*

Now it is obvious that, *in experiments with indirect stimulation*, the response/stimulus ratio *for the second pulses* can *only* be raised above that for the first pulses if for the latter it is lower than 1 1, and further, that the facilitating effect of the first pulse will become more evident if there is an increase in number of the junctions at which the transmission ratio for single impulses is less than 1 1 Because this was evidently the case during the stimulation experiments (Figs 4, 13 and 14) it may be concluded that in these experiments the fatigue has, indeed, a fairly large synaptic or presynaptic component

It is highly probable that this component is synaptic because it is inconceivable that in numerous terminal endings there occurs a gradually increasing block of the conduction for single impulses while conduction is unimpaired for impulses that arrive 25 msec later at the same terminals Moreover, it has been established that, in isolated nerve muscle preparations of frogs and rats, blocking of the impulse conduction within the terminals *occurs only at much higher rates of stimulation* (KRNJEVIĆ *and* MILEDI, 1958a, b, 1959, KRNJEVIĆ and MITCHELL, 1961) So the only explanation which remains for the difference between the transmission ratios for the two impulses evoked by a twin pulse must be that impulses due to the second pulse release more acetylcholine quanta than those due to the first, *i e* that the quantum content of the second endplate potential is larger than that of the first As to this conclusion, abundant supporting evidence may be derived from papers on neuromuscular transmission in frogs (SCHAEFER *and* HAASS, *1939*, LUNDBERG *and* QUILISH, *1953b*, DEL CASTILLO *and* KATZ, 1954, WAKABAYASHI and IWASAKI, 1962, KATZ, 1962, KATZ and MILEDI, 1966, MALLART and MARTIN, 1967, DODGE and RAHAMIMOFF, 1967, KATZ and MILEDI, 1968, MALLART and MARTIN, 1968), in toads (FENG, 1941) and in various mammals, including man (LILEY and NORTH, 1953, LILEY, 1956a, b, HUBBARD, 1963, THIES, 1965) A similar "primary" or "early" potentiation was observed in cats at the endings of Ia afferent fibres (BESWICK and EVANSON, 1954 LLOYD, 1957a, b, CURTIS and ECCLES, 1960) *of fibres in the pyramidal tract* (LANDGREN *et al*, 1962) and of those in the perforant path of the dentate area of the hippocampal gyrus (LOMO 1968) It is further present at the terminal endings of adrenergic postsynaptic fibres in guinea pigs (BURNSTOCK and HOLMAN, 1961) and cats (BROWN *et al*, 1963, KIRPEKAR and MISU, 1967)

stimuli, which occur in the ageing experiments and during repetitive low frequency stimulation (Figs 2, 4–7, 12 and 13), are not due to changes in the presynaptic inflow but that they result partly from fatigue of the muscle fibres (Figs 1, 2 and 11) and for the other part from a decrease in the number of transmitter quanta released per impulse

This decrease of transmitter output may result from a progressive decline in the amounts of readily available acetylcholine stored in the terminal endings (STRAUGHAN, 1960, OTSUKA *et al*, 1962, BROOKS and THIES 1962, HUBBARD, 1963 BRAINI *et al*, 1964, ELMQVIST and QUASTEL, 1965, THESLEFF, 1967, DODGE and RAHAMIMOFF, 1967)

Now that it has been established that repetitive indirect stimulation of isolated frog muscles with supramaximal pulses results in a lowering of the response/stimulus ratio at numerous neuromuscular junctions it may be asked whether this occurs to the same degree at the endings of all motor fibres and whether the ratio for the second pulse of a twin is raised at all junctions by the conditioning effect (3 1 3, 3 1 4) of the first pulse

This was investigated in experiments in which the muscles were stimulated indirectly with pulses of varying strength

4 RESULTS OF INDIRECT STIMULATION WITH PULSES OF VARYING VOLTAGE

4 1 EVIDENCE FOR THE EXISTENCE OF MOTOR UNITS IN WHICH A CONDITIONING PULSE IS FOLLOWED BY DEPRESSION OF THE RESPONSE/STIMULUS RATIO

Four preparations were subjected to three series of indirect stimuli given according to the usual pattern of 5 single pulses and one 25 msec twin every minute

In each of the series, the voltage range from supramaximal to subliminal was covered in 20 steps at one minute intervals The first series was given when the circulation was still intact, the second and third were started at 40 and at 80 min after interruption of the blood flow, respectively In the periods between these runs, supramaximal stimuli were applied in order to fatigue the preparations

Because the results of the four experiments were very similar,

occur in the amplitude of the electroneurogram in response to equal submaximal test pulses applied every 30 min In Fig 15b the corresponding changes are given for another 4 nerves which were repetitively excited with supramaximal single and double pulses, according to the standard pattern for stimulation (2 4)

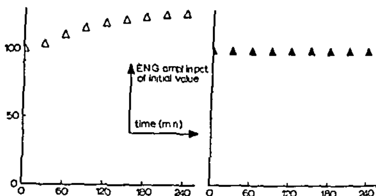


Fig 15a

Normalized averaged amplitudes of the electroneurograms in response to submaximal testpulses delivered every 30 min

Fig 15b

The same in response to supra maximal stimulation at the rate of 1 per 10 sec

From the latter experiments it appears that during repetitive stimulation of all nerve fibres there is no change in the amplitude of the ENG This strongly suggests that in the course of the previously described experiments with nerve muscle preparations there was neither a decline in the amplitude of the action potentials of individual nerve fibres, nor a decline in the number of fibres conducting an impulse in response to each pulse

Fig 15a shows that for ageing nerves which are not subjected to repetitive stimuli, the amplitude of the ENG in response to submaximal test pulses shows a steady increase As this cannot be due to an increase in the amplitude of the action potentials (Fig 15b) it must be caused by a gradual increase in the number of fibres conducting an impulse, *i e* there is an *increase* in electrical excitability of the nerve fibres during *in vitro* experiments

Therefore, it may be concluded that the decreases in amplitude of the mechanical and electrical responses to indirectly applied

response to an equal pulse applied 25 msec later It appears that the amplitude of the EMG due to single pulses varies with pulse strength according to an S shaped curve When the blood supply to the muscle is still intact (graph I) this curve closely resembles that for the amplitude of the electrical responses of the nerve (not shown)

But while after blocking of the circulation the electrical responses of the nerve to pulses of submaximal voltage increase and those due to supramaximal pulses remain the same (3 2 3, Fig 15), it follows from a comparison of the curves I–III of Fig 16 that such does *not* hold for the electrical responses of the muscle, the EMG amplitudes in response to the weakest pulses increase slightly while those due to pulses of all higher intensities diminish strongly The latter is obviously due to fatigue and ageing of the preparations (3 1 2, 3 2 2, Fig 13) On the other hand, the increases in amplitude of the EMG s in response to liminal and just supraliminal pulses must be caused by the progressive rise in electrical excitability of the nerve fibres in the course of such *in vitro* experiments of long duration (3 2 3, Fig 15a)

Because of this change in excitability of the ne
from the changes in
whether the motor u
of large diameters are equally susceptible to fatigue and to deterioration with the passage of *time* as those which have small-diameter axons and are evidently very susceptible

From a comparison of the graphs I–III *of* Fig 16 it follows that the ratio between the amplitudes of the second and *first* EMG due to a 25 msec twin pulse increases in the course of an experiment for any voltage of the twins Now, this *was to be* expected because it was concluded from the earlier *experiments on indirect* stimulation with *supra*maximal pulses that *in vitro*, there is a gradual decrease in the response/stimulus ratio *for* single pulses (3 1 3, Figs 5 and 6) which for the second pulse *of* a 25 msec twin, is partly compensated by the facilitating *after-effect of* the first pulse (3 1 3, 3 1 4, Figs 6–8), a facilitation which persists for several tenths of a second However, an unexpected fact is that during stimulation *in situ* the ratio between *the amplitudes* of the two EMG s due to the 25 msec twins is *less than* 1 1 over a narrow range of stimulus intensities This means *that the* population of

only those obtained with one of the preparations are given in Fig 16

In these experiments the contractions were not recorded as it had become evident (3 1 1) that, as a criterion for the number of muscle fibres which fire and contract in response to the nerve impulses, the amplitude of the EMG is far superior to the degree of shortening

Fig 16 shows the way in which the amplitude of the EMG due to a single pulse depends on pulse strength, together with the ratio between the amplitudes of the EMG's in response to the second and first pulses of 25 msec twins, which serves as an index for the conditioning effect of a pulse of given strength upon the

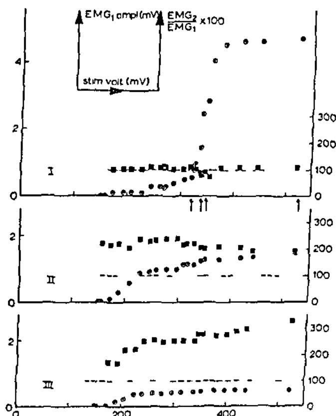


Fig 16

Plots of the amplitude of the electrical responses of a muscle to indirectly applied single pulses and of the ratio between its responses to the second and to the first pulses of 25 msec twins against stimulus strength The curves were derived from data obtained while the blood supply was intact (I) and in periods starting at 40 (II) and 80 (III) min after interruption of the circulation

that during stimulation of the nerve with supramaximal pulses, i e when each nerve fibre fires in response to every pulse, the ratio between the overall EMG's shows an early phase of facilitation

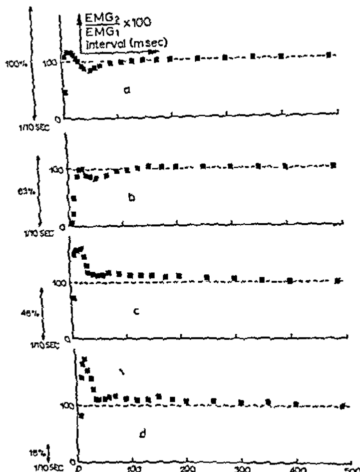


Fig 17

Ratio between the average amplitudes of the electrical responses of 4 muscles to the second and to the first pulses of indirectly applied twins, plotted against the interval between the members of the twins The data of curve a were obtained during stimulation with supramaximal pulses, those of b, c and d with pulses of lower voltages which, in the case of single pulses, evoked responses with amplitudes of about 60, 45, and 15 pct, respectively, of that of the response to supramaximal pulses.

motor nerve fibres with intermediate thresholds must contain a fairly high proportion of fibres belonging to motor units for which the response/stimulus ratio for a given pulse is lowered by a conditioning pulse applied 25 msec before

It may even be that in such units the depressive after-effect of a conditioning pulse is in reality more pronounced than is apparent from Fig 16 because in the experiments in question these units were activated together with other ones that have nerve fibres with lower thresholds and in which the first pulse of a 25 msec twin has a facilitatory after effect

In Fig 16 it is not only seen that, if stimulation is continued after interruption of the circulation, there is an increase of the EMG amplitude ratio but it further appears that after a short time there remains no appreciable difference between the ratio for pulses in this voltage range and that for pulses of other intensities (compare graphs II and III of Fig 16 with graph I) It follows that the units with conditioned depression are subject to fatigue just like the other ones

Because in the literature on neuromuscular transmission in frogs there is no indication as to the existence of motor units with conditioned depression of impulse transmission their presence was further verified and the time course of the depression was established in experiments described below

4 2 Time course of the depressive after effect of a single conditioning pulse

The time course of the depressive after effect of a single conditioning pulse on these motor units could be determined approximately in preparations where the blood supply to the muscles was *left intact throughout the experiment* Stimulation followed the usual scheme, but now the interval between the pulses of a twin was changed every minute first from the longest duration to the shortest and then in the reverse sequence This was done with supramaximal pulses and with three other intensities at which single pulses evoked EMG s having some 60, 45 and 15 pct of the maximal amplitude (comparable with those marked by the arrows in Fig 16 I)

The data obtained in four experiments were averaged, the ratio between the amplitudes of the EMG's due to the second and first pulses of a twin is pictured in Fig 17 From Fig 17a it appears

maximal pulse consists of a slight facilitation that lasts for a short period and is followed by a long lasting phase in which the second EMG is slightly depressed Upon continued stimulation the depression disappears completely while the facilitation becomes more pronounced and lasts for a longer time (compare graphs B and C of Fig 8 with graph A) It has now become evident (Fig 17) that at the start of these *in vitro* experiments the population of firing muscle fibres contains a fairly high proportion of fibres which belong to motor units showing conditioned depression From Fig 8 it may be derived that during repetitive stimulation these fibres drop out from the responses more readily than the others This is to be expected because the muscle fibres belonging to motor units with conditioned depression will drop out as soon as they do not respond any more to single pulses, while those of the motor units with conditioned facilitation contribute to the responses to twin pulses as long as they fire in response to the second pulses, although their firing to single pulses, and thus to the first pulses of twins, may have vanished long before

In addition to the explanation given in section 3 1 this, obviously, is another reason why there is, in the course of long-continued low frequency stimulation, an increasing difference between the mechanical and the electrical responses to indirectly applied single pulses and twins (Figs 2–4, 8, and 10)

We can conclude that the observations made in the classroom experiments referred to in section 1 must be explained by the fact that the preparations which have been repeatedly stimulated beforehand differ from the less fatigued ones both in the number of contracting muscle fibres (section 3) and in the proportion to which these fibres belong to the two types of motor units

5 DISCUSSION

The results of the present investigation give a fair impression as to the relative magnitude of the contributions made by fatigue and by deterioration with time to the changes occurring in the mechanical response of isolated frog muscles to the arrival of a volley of impulses along the motor nerves As was to be expected,

of the response to a second pulse which, after some 30 msec, is followed by a phase of depression lasting for some 100 msec When the voltage of the stimuli is lowered to a value at which single pulses evoke electrical responses with an amplitude of some 60 pct of the maximal one (Fig 17b), the amplitudes of the responses to the second pulses of twins are less than those to the first for a period of about 120 msec At this stimulus strength there is no initial facilitation though the depression has a minimum at about 25 msec Upon further lowering of the stimulus voltage (Fig 17c and d) there is only a phase of facilitation of the second response which is the larger in amplitude the lower are the thresholds of the activated nerve fibres It appears that for the axons with the largest diameters this facilitation lasts for more than 350 msec, with a maximum between 10 and 20 msec (Fig 17d) If only these fibres are activated there is no depression of the second EMG although the shape of the curve suggests the possible existence of a phase of relative depression which has its maximum at about 50 msec after the conditioning pulse

It may be concluded that the preparations examined consist of two types of motor units One type has motor axons of widely varied, but mainly low, threshold, its junctions respond to a conditioning impulse with an after effect that is mainly, or wholly, facilitatory and lasts for over 350 msec The other type comprises the generally less excitable nerve fibres whose endings respond to a conditioning impulse with an inhibitory after effect lasting for at least 100 msec Owing to the contribution of units of the first type to the responses (Fig 17a, b), it could not be established whether in those of the second type a phase of relative facilitation is completely absent Besides this, it has, so far, not been established by recording from single muscle fibres, whether the conditioned depression of the response/stimulus ratio in these units results from a lowering of the amplitude of the EPP's And even if this should be the case, this does not necessarily mean that such a lowering results from a decreased transmitter release

The conclusions drawn from these *in situ* experiments are pertinent to the results of the earlier experiments with supramaximal stimulation *in vitro*, where it was observed (Fig 8) that at the start of an experiment the after effect of a supra-

maximal pulse consists of a slight facilitation that lasts for a short period and is followed by a long lasting phase in which the second EMG is slightly depressed Upon continued stimulation the depression disappears completely while the facilitation becomes more pronounced and lasts for a longer time (compare graphs B and C of Fig 8 with graph A) It has now become evident (Fig 17) that at the start of these *in vitro* experiments the population of firing muscle fibres contains a fairly high proportion of fibres which belong to motor units showing conditioned depression From Fig 8 it may be derived that during repetitive stimulation these fibres drop out from the responses more readily than the others This is to be expected because the muscle fibres belonging to motor units with conditioned depression will drop out as soon as they do not respond any more to single pulses, while those of the motor units with conditioned facilitation contribute to the responses to twin pulses as long as they fire in response to the second pulses although their firing to single pulses, and thus to the first pulses of twins may have vanished long before

In addition to the explanation given in section 3 1 this, obviously, is another reason why there is, in the course of long-continued low frequency stimulation an increasing difference between the mechanical and the electrical responses to indirectly applied single pulses and twins (Figs 2–4 8, and 10)

We can conclude that the observations made in the classroom experiments referred to in section 1 must be explained by the fact that the preparations which have been repeatedly stimulated beforehand differ from the less fatigued ones both in the number of contracting muscle fibres (section 3) and in the proportion to which these fibres belong to the two types of motor units

5 DISCUSSION

The results of the present investigation give a fair impression as to the relative magnitude of the contributions made by fatigue and by deterioration with time to the changes occurring in the mechanical response of isolated frog muscles to the arrival of a volley of impulses along the motor nerves As was to be expected,

the electrical excitability of the muscle fibre membranes, and the contractility of the actin myosin filaments, are influenced by both (3 2 1) Evidence was presented that during long continued stimulation there must be a fairly large decline in the number of acetylcholine quanta released by each impulse, but that such a decrease hardly results from ageing alone (3 2 2)

It further appeared that this synaptic fatigue occurs earlier at the endings of the high-threshold fibres than at those of the more excitable axons (4 1)

After interruption of the blood supply the acetylcholine stores cannot be replenished, as a result of anoxia (GRIEVE, 1958, KRNJEVIĆ and MILEDI, 1959, BROOKS and THIES, 1962) and of lack of glucose (STRAUGHAN, 1960) and of choline (BIRKS and MACINTOSH, 1957, 1961, BEANI *et al*, 1964) For that reason, the more rapid decline of transmitter release at the endings of the high threshold nerve fibres must be due to their initially having smaller stores of readily releasable acetylcholine (BIRKS and MACINTOSH, 1957, ELMQVIST and QUASTEL, 1965, THESLEFF, 1967), or to a greater susceptibility to anoxia of the mechanism that effects the coupling between depolarization of the presynaptic membrane and release of acetylcholine As to the latter, there is abundant evidence to support the theory of KATZ and MILEDI (1966, 1967, 1968), according to which the impulses arriving at the nerve endings trigger the release of free calcium ions from a store of bound calcium within the presynaptic membranes These calcium ions travel inwards and combine with specific sites for transmitter release located on the inside of these membranes, which then become suitable for the attachment of presynaptic vesicles and for the expulsion of their contents

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Seen in this light, and assuming that the stores of readily releasable acetylcholine are the same in all fibres, then the fact that

fatigue of transmitter release occurs more rapidly at the endings of the high threshold fibres may result either from a release of more calcium ions per impulse, or from a slower removal of the released ions from the specific sites for transmitter release In both cases the sites will be occupied for a longer period than those of the low threshold fibres with the result that the conditioning action of one impulse upon the response to a closely following one should be more pronounced and of longer duration at the endings of the high threshold fibres As to this conditioning action, recent evidence has supplemented the calcium concept with the hypothesis that the cooperative action of four calcium ions is necessary for the release of a transmitter packet from a given site (DODGE and RAHAMIMOFF, 1967, KATZ and MILEDI, 1968, RAHAMIMOFF, 1968) and that the facilitation of transmitter release in the case of a test pulse results from the presence of residual calcium at the specific sites According to this attractive hypothesis, the time course of facilitation after a conditioning impulse would reflect the removal of these remaining calcium ions from the release sites From the present investigation, however, it appears that though there is indeed such a phase of facilitation in numerous motor units of the frog, it is absent in others This can hardly be reconciled with the calcium hypothesis Moreover, this hypothesis does not account for the fact that in mammalian preparations at body temperature there is only a phase of conditioned depression, which can be converted into a phase of facilitation by lowering of the temperature (LUNDBERG and QUILISH, 1953, HUBBARD, 1963, HUBBARD and SCHMIDT, 1963, THIES, 1965) In this respect it should be recalled that facilitation of transmitter release has often been attributed to the presence of an increased amount of readily releasable transmitter, which would result from the "mobilization" of transmitter packets from a "mobilization store" towards the presynaptic membrane (ECCLES, 1957, DE ROBERTIS and VAZ FERREIRA, 1957, CURTIS and ECCLES, 1960, BROOKS and THIES, 1962, HUBBARD and WILLIS, 1962, HUBBARD, 1963, HUBBARD and SCHMIDT, 1963, ELMQVIST and QUASTEL, 1965, THESLEFF, 1967) According to some papers (LLOYD, 1949, KATZ, 1962, TAKEUCHI and TAKEUCHI, 1962) this mobilization is accompanied by a hyperpolarization of the presynaptic membranes and an increased amplitude of their action potentials, which has led to

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Seen in this light and assuming that the stores of readily releasable acetylcholine are the same in all fibres then the fact that

1941, HUBBARD, 1963, HUBBARD and SCHMIDT, 1963, ORKAND, 1963, THIES, 1965), this has to be investigated more fully

Anyhow, it seems that the most important observation made during the present investigation is that in frog muscles that are stimulated indirectly, either *in vitro* or *in situ*, the response/stimulus ratio of numerous muscle fibres is lower for a conditioning pulse than it is for a closely following test pulse of the same voltage Because each of these pulses will generate one impulse in every nerve fibre the threshold of which is exceeded by the stimulus voltage, it must be concluded that, in these circumstances, for the frog s twitch system the "safety factor" for neuromuscular trans mission is rather low and that many myoneural junctions do not function as ' simple relays" This is contrary to what has repeatedly been asserted (FATT and KATZ, 1951, KATZ, 1962, 1966 BROOKS and THIES, 1962, THESLEFF, 1967), on the other hand, evidence supporting our conclusion may be found in papers by WAKABAYASHI and IWASAKI (1962), and by ORKAND (1963) Therefore, it is to be expected that in the *intact* frog the responses of the twitch fibres to the firing pattern of their motoneurones will strongly be influenced by fatigue facilitation and depression of impulse trans mission and that, as a consequence, their contractions will be determined by both the rate and the pattern of motoneurone discharge

SUMMARY

Electrical and mechanical recordings were made from isolated frog muscles during direct or indirect stimulation with single pulses and with twins

It appears that the decrease of the responses during indirect low frequency stimulation (3 1 1 3 1 2) partly results from a lowering of the response/stimulus ratio of the twitch fibres, which is due to a decrease in the transfer ratio at their myoneural junctions (3 1 3)

This decrease in transfer ratio during repetitive stimulation results from the increase in number of the stimuli applied and not from the passage of time (3 2)

If the ratio between the amplitudes of the responses to the second and first pulses of twins is used as a criterion for changes in the transfer ratio at motor endplates it appears that there are two [illegible]
the frog s twitch system In unit [illegible]
followed by facilitation of the re[illegible]
while this response is depressed [illegible]

The differences between the cor[illegible]

the assumption that the short time facilitation must be closely related to the long lasting phenomenon of post tetanic potentiation (FENG, 1941, LLOYD, 1949, LILEY, 1956c, CURTIS and ECCLES 1960, HUBBARD and WILLIS, 1962a, b, GAGE and HUBBARD, 1966), although the existence of such a relation is firmly denied in other papers (LILEY and NORTH, 1953, ECCLES *et al*, 1962, HUBBARD, 1963, HUBBARD and SCHMIDT, 1963)

If, on the other hand, facilitation takes its origin from residual attachment of calcium ions to the release sites, then it must be expected that the results *reported in section 4 of the present paper* will point in the direction of a strong positive correlation between *the rate of development of fatigue at myoneural junctions and the* degree of facilitation after a conditioning pulse Although such a relation may have been masked by the presence of motor units with conditioned depression (4 1, 4 2), the very existence of such units cannot be explained on the basis of the calcium hypothesis for facilitation And even if in these units too the depolarization-release coupling is effected by calcium, then the differences in the response to test pulses between the two types of units must have another cause, such as different properties of the postsynaptic elements

It is well known that in frogs the motor units belong to two sharply separated types, one with large axons and phasic muscle fibres while the other consists of small axons innervating tonic muscle fibres (KUFFLER and VAUGHAN WILLIAMS, 1953a, b KUFFLER 1955 BURKE and GINSBORG 1956a, b, BURKE, 1957, ORKAND, 1963, COSTANTIN *et al* 1967)

In several respects this subdivision of the frog s motor units does not coincide with that derived from the present investigation (4 2), the motor units showing conditioned depression of their transfer function do not differ sharply from the others in the excitability of their nerve fibres, they exhibit a conditioned depression instead of the mere absence of facilitation which has been reported for the small nerve slow muscle fibre system, and it appears that they are not less susceptible to fatigue than motor units with axons of larger diameter, while the opposite has been reported to hold for motor units with *s n j p* 's (KUFFLER and VAUGHAN WILLIAMS, 1953b, WUERKER *et al*, 1965)

From the present investigation it further appears that motor units of the frog differ widely in the relative degrees of facilitation and depression, even in such measure that either one or the other may be absent Because this is conflicting with earlier reports, according to which both phases occur in every motor unit (FENG,

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single pulses and twins (I, 3 1 1) result both from 'pure' contraction summation (3 1 5) and from recruitment of muscle fibres (3 1 3, 3 1 4).

The fact that, already at the onset of stimulation *in vitro*, the response/stimulus ratio is lower than 1 1 for part of the twitch fibres (3 1 2, 3 1 3) suggests that in the intact frog the response of these fibres to a given presynaptic inflow will be influenced by fatigue, and by facilitation and depression of their myoneural junctions

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A METHOD FOR THE DETERMINATION OF SYSTEMIC ARTERIAL COMPLIANCE IN MAN

BY

J G DEFARES AND H J VAN DER WAAL

CONTENTS

1 INTRODUCTION

Since the arterial blood pressure and the impedance against which the left ventricle performs work are determined by both the total peripheral resistance (*TPR*) and the systemic arterial compliance the estimation of the latter quantity possesses a medical and physiological significance comparable to that of *TPR*

As arterial compliance cannot directly be measured certain simplifying assumptions must be introduced as in the case of *TPR* measurement For a full discussion of the theoretical background of the method the reader is referred to an earlier paper (DEFARES, OSBORN and HARA, 1963)

2 METHOD

2 1 PRINCIPLE

For the present purpose the arterial system is treated as a simple lumped system, consisting of a resistive element (*TPR*) and a compliance (*C*) and driven by a periodic forcing

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systolic and end-diastolic pressures? In the above mentioned experiment SPENCER and DENISON (l c) found that $p_s - p_d$ as measured from the RC network forced by the (transduced) flow in the ascending aorta differed by less than 5 % from that directly measured in the ascending aorta This represents direct evidence that the physical factors responsible for the deviation of the actual shape of the aortic pressure curve during diastole from a simple exponential decay curve have little effect on p_s and p_d in the ascending aorta Computer results based on a complex model of the circulation led to a similar conclusion (DEFARES *et al* , 1963)

Since for the purpose of estimating C one should obtain $p_s - p_d$ at a site where no or little distortion due to filter network effects occurs, the ideal location for measuring p_s and p_d would be in the ascending aorta while the femoral artery would be unacceptable The rise, in the legs, of p_s relative to the p_s value of the central pulse is not found in the arms (e g WARNER, 1957), a phenomenon which, in terms of the formally valid resonant network model, is partly explained by the fact that the lumped compliance of the abdominal aorta and its branches is smaller than the lumped compliance of the aortic arch and its branches (SPENCER and DENISON, l c)

This justifies the practice of using the arm as a convenient site for the measurement of p_s and p_d as required for solving Eq (3) for C

2 2 MEASUREMENTS

Blood pressure was measured in the supine position by means of the Riva Rocci method using the point of muffling as index of diastolic pressure Total peripheral resistance, R, was estimated from the relation $R = \bar{P}/\dot{Q}$, where $\bar{P}$ is mean arterial blood pressure and $\dot{Q}$ is cardiac output In this formula systemic venous pressure is neglected, and $\bar{P}$ is estimated by the standard procedure of adding one third of the diastolic pressure value to the diastolic pressure Cardiac output was measured by the indirect Fick method previously described (DEFARES *et al* , 1961, BRANDI, 1961)

The diastolic time interval, t_d is estimated from the ECG according to Peo Gribbe's criteria as recommended in the Handbook of Physiology (BRECHER and GALLETI, 1963) i e the time interval between the end of the T-wave of the n^{th} cycle and the beginning

SPENCER and DENISON (1963) have shown that when, in the dog, the electrical equivalent of the Windkessel model is pulsed by a current transduced from the flow in the ascending aorta the actual pulse pressure in the aortic arch deviates in several details from the "pressure" produced by the model the actual pressure has (1) a superimposed 3 to 6 cps oscillation from midsystole throughout diastole, and (2) a more prominent "incisure" marking aortic valve closure and a more abrupt rise often with an anacrotic wave In addition the Windkessel model fails to explain the changes in shape occurring along the arterial line In order to simulate detail (1) one may introduce a resonant network filter (WARNER 1957)

However, for our present purpose it is neither necessary nor indeed desirable to introduce complexity in the model to account for details which are irrelevant for the purpose at hand

The simple RC model leads to a pressure time curve during diastole of the form

$$p = p_0 \exp\left(\frac{-t}{RC}\right) \tag{1}$$

For a duration of diastole t_d we have

$$p_d = p_0 \exp\left(\frac{-t_d}{RC}\right) \tag{2}$$

where p_d is the end diastolic pressure and p_0 represents the pressure at the onset of diastole i e the end of the previous systole In practice, p_0 may be approximated by the end systolic pressure p_s or

$$p_d - p_s \exp\left(\frac{-t_d}{RC}\right) \tag{3}$$

Equation (3) gives the formula for estimating the arterial compliance C Knowledge of (end) systolic pressure (end) diastolic pressure duration of diastole and peripheral resistance is thus required

An important question must be considered before this approach can be accepted as a reasonable indicator of overall arterial compliance To what extent does the use of the simple RC circuit rather than a more complex network affect the values of simulated peak

error of a single observation, 0 053, it follows that it is roughly 10 %, while the root mean square error of a single observation is about 15 % This computation implicitly and conservatively assumes that during the period of observation the "true" compliance remains unchanged

A more basic evaluation of the error in C is obtained by evaluating its dependence on the errors in the sub quantities, i e the quantities appearing on the right hand side of eq (4)

$$C = \frac{t_d}{R(\ln p_s - \ln p_d)} \tag{4}$$

We have

$$\frac{\delta C}{\delta t_d} = \frac{1}{R(\ln p_s - \ln p_d)}$$

$$\frac{\delta C}{\delta p_s} = \frac{-t_d}{p_s R}(\ln p_s - \ln p_d)^{-2}$$

$$\frac{\delta C}{\delta p_d} = \frac{t_d}{p_d R}(\ln p_s - \ln p_d)^{-2}$$

$$\frac{\delta C}{\delta R} = \frac{-Rt_d}{R^2(\ln p_s - \ln p_d)}$$

Hence we obtain, after some manipulations

$$\left.\begin{aligned} \text{var } C = R^{\;2}(\ln p_s - \ln p_d)^{-2}\{\text{var } t_d + t_d^2 R^{-2}\text{ var } R + t_d^2 \\ (\ln p_s - \ln p_d)^{-2}(p_s^{-2}\text{ var } p_s + p_d^{-2}\text{ var } p_d)\} \end{aligned}\right\} \tag{5}$$

Let us first consider a theoretical case, assuming a (root mean-square) error of 10 % in all subquantities Choosing $p_s = 120$, $p_d = 80$, $R = 1$, $t_d = 1$ and assuming, as stated, var $p_s = 0\ 01\ p_s^2$, var $p_d = 0\ 01\ p_d^2$, var $R = 0\ 01\ R^2$, var $t_d = 0\ 01\ t_d^2$, we obtain using eqs (4) and (5),

$$\frac{\sigma(C)}{C} = 0\ 317,$$

or a root mean square percentage error of about 30 %

of the $(n+1)^{th}$ is taken as an estimate of t_d [1]). "Healthy" subjects (♂ and ♀) between 18 and 86 years were used in this study.

Measurements were taken at rest in the supine position with the exception of the young male subjects who were studied at 'rest' in the sitting position [2]). Preliminary results indicate that C values are little affected by position.

2.3 ACCURACY

2.3.1 *Random error*

The accuracy of the method was assessed by performing serial measurements on a single individual in a single sitting (3 hours). The subject was a 76 year old male, "healthy" except for a moderate degree of hypothyroidism commensurate with his age. The results are given in Table 1.

TABLE 1

Measurement number	P_s mm Hg	P_d mm Hg	t_d sec	Q l/min	C ml/mm Hg
1	156	94	0.75	2.26	0.49
2	158	96	0.75	2.16	0.44
3	158	96	0.75	2.86	0.59

It follows from the three estimates of C given in Table 1 that in Trimmer's notation (TRIMMER 1950) the root mean square error ΔC_{rm} is 0.076 ml/mm Hg, the probable error ΔC_{rp} is 0.052 ml/mm Hg and the probable error in the arithmetic average ΔC_{rpa} is 0.0294. Expressing the result of the measurement as recommended by TRIMMER, as $C_{aa} \pm \Delta C_{rpa}$ where C_{aa} denotes the arithmetic mean, we obtain $C = 0.507 \pm 0.02$ ml/mm Hg. From the probable

---

[1]) Since the measurements used are indirect they may be subject to some systematic error. Since we are mainly concerned with relative values the possible presence of some systematic error is inconsequential. The best indirect measure of t_d continues to be a subject of some controversy. A disadvantage of the Gribbe criterium is that the beginning of the T wave cannot be defined in some cases with elevated $S-T$ segment.

[2]) Since the first old subject tested did not tolerate the sitting position the supine position was finally chosen. This decision was made after the experiments in the young male subjects had been completed.

p 193 of the paper of DEFARES *et al* (1963) Here we shall discuss only the problem of the contribution of inertia, reflections and elastic and geometric taper on the arterial pressure curve as related to our problem

First it should be noted that, contrary to the older physico-mathematical theories, reflections are only of secondary importance in explaining the changes of the arterial pressure wave on its way towards the periphery In a formal way this was demonstrated by WARNER (1957), who was able to reproduce the shape of the pulse wave by means of a resonant network model

FICH and WELKOWITZ (1966, 1967) have shown that pressure and flow waveforms in the aorta can be calculated by assuming geometric and elastic taper and neglecting reflection

The assumption that reflection can be neglected was directly tested *in vivo* in the following ingenious way The hearbeat of dogs was stopped by application of electric pulses and the recovery was observed The first recovery wave, substantially unaffected by reflection was found to have the same waveform as the succeeding waves

Measurements were made at various points along the aorta The striking identity between the transient and the steady state waveforms offers direct experimental proof that the role of reflection in determining the waveform is unimportant

As has been demonstrated by these authors and others (e g TAYLOR 1964) the same holds for the contribution of inertia It is now well established, as was first suggested by SCHMITT (1943), and recently convincingly shown by NOORDERGRAAF (1964), TAYLOR (1964), and FICH and WELKOWITZ (1966, 1967) that the dispersive characteristics of the arterial system caused by elastic and geometric tapers are largely responsible for the observed waveforms Harmonic analysis of arterial pressure waveform shows that in mammals *all* components increase in amplitude towards the periphery This overall amplification, first noted by SCHMITT and amply documented by TAYLOR, can only be adequately explained in terms of a peripheral decrease in compliance due to elastic and/or geometric taper In fact, it can be shown (STREETER, 1961) that pressure pulses travelling in a non uniform transmission line of this kind undergo an amplification proportional to the square root of the local characteristic impedance In a uniform

The actual variances in the sub quantities as obtained from a single experiment are, however, found to be much lower namely var $t_d \approx 0\ 002$ var $p_s = 7\ 29$, var $p_d = 3\ 96$ (all based on 10 observations), var $R \approx 0\ 002$ (based on data in Table 1) With $p_s = 157$, $p_d = 94$, $t_d = 0\ 72$ and $R = 2\ 86$ Eq (5) yields

$$\text{var}\, C = 7\ 456 \times 10^{-4}$$

or 0 027, i e a root mean square percentage error of 5 %

Since R is itself a derived quantity whose computation is based on standard simplifying assumptions, and because confidence limits of the variance of R are wide, since our value of var R is based on only three values of R, this estimate of var C only reflects an estimate of its order of magnitude It is felt that a conservative estimate of the error of a single observation is obtained by simply averaging the results of the 'empiric' value and the value estimated by means of Eq (5) which yields for the root mean square percentage error of a single observation a value of $(15+5)/2 = 10$ %

2 3 2 *Determinate error*

The assessment of total error depends on knowledge of the determinate error, a factor which in the indirect determination of physiological quantities remains largely unknown and can be estimated *only from (a) theoretical considerations and (b) the* degree of correspondence between the results of several independent *methods Since there are no other reliable methods to estimate C in vivo* (the pulse propagation method measures the local elasticity *of the arterial wall) determinate error can only be estimated on* the basis of theoretical analysis

The situation is closely analogous to the state of the art in biological tracer experiments where the validity of results is critically dependent on the compartmental model and on the nature of the operational definitions

The major theoretical consideration supported experimentally by SPENCER and DENISON has been presented above It is neither feasible nor necessary fully to discuss the theoretical background here [1]) The reader is particularly referred to the discussion on

[1]) The method proposed by R M GOLDWYN and T B WATT Jr IEEE Transactions on Bio Medical Engineering vol BME — 14 p 11 is of interest, but subject to severe errors

It is of interest to note that in contradistinction to most waveforms obtained in experimental animals, the form of the pressure curve during diastole in the ascending aorta (and arch) in humans closely approximates a simple exponential decay at normal heart rates

This is illustrated in Fig 1, representing pressure[1]) in the ascending aorta of a 58 year-old woman with normal aortic valves (PORJÉ, 1964) This fact lends further support to the view that, in humans, the central pressure is little affected by inertia, reflection and other "secondary" influences including the elastic non uniformity of the transmission line, theoretically the major "secondary factor" affecting the form of the central pulse

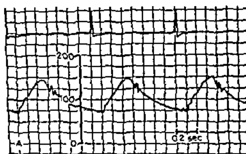


Fig 1

Ascending aortic pressure in a 58 year old woman with normal aortic valves. (after I G PORJÉ, 1964)

There is one further theoretical point that requires careful consideration It is well established that the arterial system still contains blood when the pressure in the system is reduced to (near) zero after death

This fact implies that C is not a constant (DEFARES *et al*, 1963) *It is customary in circulation physiology to define compliance as* dv/dp rather than v/p, where v is volume and p is pressure Above, *compliance* has been given as dv/dp which is quite adequate for most *practical* purposes, dv/dp being equivalent *to* v/p when compliance is a constant In the theoretical discussion below we shall

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[1]) Measured by Dr Porjé with the Roos double lumen catheter and a pressure transducer system giving virtually correct reproduction of amplitude and phase characteristics for frequencies up to 12 cps

transmission line this effect does not occur The finding (TAYLOR, l c) of a peripheral increase of the collagen/elastin ratio in the aorta and arteries lends further support to the concept of elastic taper.

As demonstrated theoretically by Taylor the presence of this kind of elastic non-uniformity has the following two consequences

a) due to an approximate matching with the peripheral terminations of the system there are only small reflected components, which keeps the input impedance relatively constant over a wide range of frequencies
b) *at physiological frequencies the heart does not "see" the high* terminal impedance but $Z_{0\,0}$ which is small

Since (*see e g* WARNER, *1957*, REMINGTON, *1952*, SPENCER and DENISON, 1963) the results of measurements at the (upper) arm (Riva-Rocci) approximate those obtained by direct measurement at the aortic arch and since reflections are of negligible importance, it follows from the above considerations that C as estimated by our method approximates the compliance of the "soft" end of the arterial transmission line, which from the "view-point" of the heart (generator) is the most "visible" part at physiological frequencies In terms of Dow's, Wetterer's and Remington's terminology we may say that our method estimates the average compliance of the widest part of the "funnel" (arch, thoracic aorta), although any precise geometric anatomical delineation is (as in the case of peripheral resistance) devoid of meaning

The present method thus measures the "central" systemic arterial compliance rather than the overall systemic arterial compliance Here the boundaries of "central" are no more sharply defined than in the concept or "central blood volume" Since our method is applied to humans it is of interest to inquire whether the human *arterial system represents a non uniform transmission line in the* sense discussed above LUCHSINGER (l c) has provided convincing support for this view by showing, using normal unanesthetized subjects, that the (first eight) harmonics of the pressure modulus all show an overall increase along the aorta down to the iliac artery, whereas the respective phase angles show a decrease with distance, a behaviour "consistent with a travelling wave in a non uniform system"

TABLE 2

For meaning of symbols see legend

n	Sex	a	p_s/p_d mm Hg	$\dot{Q}$ l/min	R mm Hg/ml/sec	C ml/mm Hg	S mm Hg/ml
1	f	19	108/78	3 1	1 7	1 3	0 77
2	f	25	98/72	4 0	1 2	2 8	0 36
3	f	23	96/62	3 5	1 3	1 7	0 59
4	f	29	100/80	4 2	1 3	2 3	0 43
5	f	26	88/70	4 0	1 1	2 9	0 34
6	f	24	90/74	4 1	1 2	3 2	0 31
7	m	18	122/80	10 8	0 5	3 4	0 29
8	m	18	130/88	10 9	0 5	2 6	0 38
9	m	21	118/78	8 5	0 6	1 7	0 59
10	m	21	142/86	7 4	0 9	1 0	1 00
11	m	21	110/76	8 9	0 6	2 7	0 37
12	m	22	114/84	9 0	0.7	1 8	0 56
13	f	22	92/78	6 3	0 8	4 9	0 20
mean		22		6 5	0 96	2 5	0 48
n	Sex	a	p_s/p_d mm Hg	$\dot{Q}$ l/min	R mm Hg/ml/sec	C ml/mm Hg	S mm Hg/ml
1	f	78	180/77	2 7	2 6	0 3	3 33
2	f	74	176/91	3 8	1 9	0 4	2 50
3	f	76	166/91	1 8	4 0	0 2	5 00
4	f	86	169/92	3 4	2 1	0 6	1 67
5	m	74	142/94	3 1	2 1	0 7	1 43
6	m	73	149/109	2 8	2 6	1 3	0 77
7	m	73	156/82	4 0	1 6	0 7	1 43
8	m	81	180/100	2 1	3 6	0 3	3 33
9	m	83	168/89	2 3	3 0	0 3	3 33
10	m	75	193/95	1 7	4 5	0 2	5 00
11	m	73	155/97	2 3	3 1	0 6	1 67
12	m	78	151/78	6 4	1 0	1 2	0 83
13	m	74	147/89	3 0	2 2	0 7	1 43
mean		77		3 0	2 6	0 58	2 44

n=subject code a=age p_s/p_d=arterial pressure $\dot{Q}$=cardiac output R=total peripheral resistance C=arterial compliance (dv/dp), $S=1/C$

seen that whereas the mean value of total peripheral resistance of the aged group is 2 75 times that in the young group, the mean

define compliance as $C^* = v/p$ If C^* is a constant, we obtain $v=0$, which is contrary to the fact that the arterial system still contains blood when p is zero The simplest way to account for the requirement $v>0$ for $p=0$ is to assume

$$C^* = (M/p) + N \tag{6}$$

where M and N are positive constants

Substituting Eq (6) in the general expression $v=Cp$, we obtain $v=M+Np$ For $p=0$, $v=M$ which expresses the fact that the "unstretched" arterial system still contains blood

It is easily seen that C (dv/dp) equals N Since Eq (6) must logically hold it is essential to investigate theoretically the validity of our assumption that (ignoring "secondary effects") the arterial $p-t$ curve during diastole shows a simple exponential decay with time-constant NR ($\equiv CR$) Assuming relation (6) and $R \equiv$ constant, neglecting inertance, elastic non uniformity, and other secondary effects, the differential equation for the diastolic period (in the absence of forcing) is

$$NR\frac{dp}{dt} + p = 0 \tag{7}$$

in exact agreement with Eq (1) with $C=N$

This means that our assumption of the validity of Eq (1) is consistent with the fundamental requirement expressed by relation (6) An important practical consequence of the result expressed by Eq (7) is that the value of C as determined by Eq (2) is unaffected by the "residual volume" in the arterial system This is of importance since aortic "dead" volume increases with age (*dilatatio aortae*) despite *increased stiffness of the wall* Our method estimates $C = dv/dp$, a value which is independent of dead volume M

3 RESULTS

The "rigidity" of the systemic arterial system is known to increase with age, a fact well established by autopsy studies

Thus, a comparison between aged subjects and young adults should provide a *critical test for the usefulness of the method* The results are shown in Table 2 The mean age of the young group is 22 years, while that of the aged group is 77 years It may be

"young" ECG's and BCG's require rather sophisticated statistical techniques In contrast, the old-young ratio is about 5 0 with respect to arterial elastance, S, with very little overlap of the two groups With advancing age there is, besides the increased hardening (stiffness) of the aorta and the arteries an accelerated development of atheromatous plaques Although the ratio hardening plaques differs from one case to another, both may be viewed as aspects of the age related process of arteriosclerosis The present method gives an estimate of the overall degree of rigidity of the arterial system and answers the question as to the degree of arteriosclerosis (not atherosclerosis) in the living individual [1]) It is expected that the measurement of both TPR and C (S) should give a far more accurate picture of cardiovascular pathology than the mere measure ment of blood pressure As a hypothetical example (DEFARES *et al*, 1963) we note that a blood pressure reading of 270/60 (which some may find reassuring since diastolic pressure is low), reflects, at normal cardiac output, a doubling of peripheral resistance combined with a ten fold increase of arterial 'stiffness', S The prognostic significance of high S values, the influence of S on cardiac work (see TAYLOR l c) its correlation with blood lipid factors (e g atheromatic index) and collagen metabolism (hydroxyproline), its influence on ballistocardiogram patterns, etc are some of the specific problems for future exploration For the present, however, two conclusions emerge

a) the present method offers a sensitive means to detect changes of a fundamental cardiovascular parameter, which has hitherto resisted attempts to measure it *in vivo* (the pulse wave trans mission method estimates the modulus of elasticity of a single artery)

b) arterial elastance undergoes profound changes with aging, a fact suggesting that arterial elastance, S, may be used as a sensitive index of the "biological age" of the cardiovascular system and possibly of the whole organism, as suggested by the age-old adage "man is as old as his arteries"

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[1]) The term 'arteriosclerosis was introduced by LOBSTEIN (1833) to refer to a state of hardening of the arteries The term "atherosclerosis' refers to the presence of 'atheroma in the sense defined by MARCHAND (1904) For a detailed discussion of these concepts see MOSES (1963)

value of the arterial elastance, S, (reciprocal value of the compliance) in the aged group is 5 0 times that in the young group [1])

It is of some interest to note that, as may be seen from Table 2, the smallest stiffness ratio between members of the old and the young group is about 0 75 while the highest is 25 0 The old subject with the lowest S value of his group (0 77) still has a value 60 % above the mean of the young group, while the highest value in the aged group (5 0 mm Hg/ml in subjects 3 (female) and 10 (male)) is ten times the mean value of the young group

As can be statistically shown the male-female distribution in the young and in the aged group can not account for these differences, which on the basis of available evidence are an age dependent effect It may be mentioned in passing that the mean S value of a group of 13 young females (mean age 23) was found to be 0 44 as against 0 48 in the "mixed" young group given in Table 2 Further, it may be noted that if we compare the four old females with the first four young females in table 2 (arbitrary choice) we obtain a mean "old" value of 3 13 against a mean "young" value of 0 54 Their ratio is 5 8 These facts indicate that the slight preponderance of females in the young group in Table 2 does not increase the difference due to an *a priori* possible extra low elastance of the young female as compared with the young male Since it is not the purpose of this paper to determine compliance characteristics of young and of old individuals (this forms the subject of another paper) we shall omit a more detailed statistical analysis of these data

The presence of such wide differences among normal subjects of different age groups and the availability of a fairly accurate method to estimate this important circulatory quantity, offer wide possibilities for further exploration No other known cardiovascular quantity (including *TPR* blood pressure cholesterol and other lipids, cardiac output ECG, ballistocardiogram etc) undergoes such profound alterations with aging as the elastance changes observed in this study The "old young" ratios of *TPR*, blood pressure, cholesterol, and cardiac output are (roughly) 2 5, 1 5 1 5 and 0 5 respectively, while discrimination between "old ' and

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[1]) Using Student s test it can be shown that this difference is highly significant (actually use of statistical technique is pedantic here)

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SUMMARY

A method for measuring arterial compliance (1/elastance) in humans is presented The theoretical basis is discussed and it is shown that inertia reflections and other transmission line effects can be neglected The significance of elastic and geometric taper and of "dead" volume are discussed The accuracy of the method is of the order of $\pm 10\%$ The method is simple and causes no inconvenience to the subject It is shown that the mean value of arterial elastance is five times higher in a group of aged individuals (mean age 77) than in a group of young persons (mean age 23) The relatively high accuracy (and reproducibility) of the method combined with the profound age related changes of arterial elastance found even among "normal" persons suggests that the present method offers a useful tool for clinical and physiological studies in man

ACKNOWLEDGEMENT

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METHODS

Male albino rats, weighing about 210 g, were killed by dislocating the spinal cord The brain was removed quickly and chilled on an ice-cold plate The cortex (defined by GLOWINSKI and IVERSEN, 1966) was used to prepare a 0 25 M sucrose homogenate (10 pCt w/v) enriched with 10 μ/M $CaCl_2$ (recommended by DE ROBERTIS *et al* 1963) Homogenisation in a Potter Elvehjem homogenizer with teflon pestle was carried out within one minute at 3000 rpm, clearance 0 10 mm, three up and down strokes Working temperature at all subsequent operations was 0–4° C

DIFFERENTIAL FRACTIONATION

The, slightly modified, method of RODRIGUEZ DE LORES ARNAIZ and DE ROBERTIS (1964) was applied Cortex homogenate centrifuged at 900 g Phywe Eispirouette for 10 min produced a sediment, which, resuspended in 0 25 M sucrose, was considered to be the nuclear fraction (NUC)

Next, centrifuging at 10 000 g for 20 min sedimented a pellet which, resuspended in 0 25 M sucrose, is referred to as crude mitochondrial fraction (MIT), consisting of myelin, nerve endings, synaptic debris and genuine mitochondria This MIT fraction was subjected to density gradient centrifugation The final particulate fraction obtained by centrifuging with angle rotor at 105 000 g for 60 min in Phywe P 30 K was called microsomal fraction (MIC) Remaining was the supernatant fraction (SUP)

DENSITY GRADIENT CENTRIFUGATION

The procedure of DE ROBERTIS *et al* (1962) was followed The crude mitochondrial fraction (MIT) was layered on a sucrose gradient with the steps 0 8 M, 1 0 M, 1 2 M, 1 4 M all containing 10 μM $CaCl_2$ With the swing-out rotor of Phywe P 30 K centrifuge at 130 000 g for 60 min the submitochondrial fractions myeline (MIT-A), membranes, synaptic debris (MIT-B), nerve endings (MIT C, D) and mitochondrial pellet (MIT-E) were separated (Fig 1) The distinct layers were collected by sucking with a syringe

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INFLUENCE OF THIAZINAMIUM [1]) ON SUBCELLULAR p NITROPHENYLPHOSPHATASE IN RAT CEREBRAL CORTEX

BY

C DE WAART AND D H VAN DEN EIJNDEN

INTRODUCTION

Among the earliest authors who described the influence of pheno thiazines on brain preparations were DESCI and MÉHES (1957) They observed the uncoupling of the oxydative phosphorylation and the inhibition of oxygen uptake and suggested this phenomenon as an explanation for the narcobiotic effect *in vivo*

More recently other inhibitive actions of the phenothiazines were studied by LØVTRUP (1963, 1964, 1965) on DNP and Mg^{++} activated ATP ase in liver and brain mitochondria, by JARNEFELD (1962) and by DAVIS and BRODY (1966) on Na^+ K^+ activated ATP ase in brain microsomes and by JUDAH and AHMED (1964) on lipoprotein preparations from brain microsomes In all these cases the inhibition was correlated to changes in membrane permeability Reports on actual stimulation of enzyme activity by phenothiazines are few Löw (1959) and LØVTRUP (1965) could demonstrate increased enzyme activity only at drug concentration below 0 1 mM

In previous communications (1966 1967) we reported an in creased activity of *p* nitro phenylphosphatase at concentrations of phenothiazines up to 10 mM The present paper will describe this effect on rat brain fractions prepared by differential and density gradient centrifugation

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1) Thiazinamium (Multergan) and other phenothiazine derivatives were kindly supplied by 'SPECIA", Société Parisienne d Expansion Chimique S A Paris

Fig. 1

homogenate

900 g for 10 min
- NUC
- supernatant
  - 10 000 g for 20 min
    - supernatant
      - 105 000 g for 60 min
        - supernatant SUP
        - MIC
    - crude mitochondrial fraction MIT pellet layered on discontinuous sucrose density gradient
      - 130 000 g for 60 min
        - 0.25 — MIT–A
        - 0.8 — MIT–B
        - 1.0 — MIT–C
        - 1.2 — MIT–D
        - 1.4 M sucrose — MIT–E

a) scheme for density gradient fractions

crude mitochondrial fraction MIT pellet resuspended in 0.032 M sucrose $+10\ \mu M\ CaCl_2$

osm MIT

10 000 g for 20 min
- supernatant
  - 105 000 g for 30 min
    - supernatant M_3
    - M_2 pellet
- M_1 pellet resuspended in 0.25 M sucrose layered on discontinuous sucrose density gradient
  - 130 000 g for 60 min
    - 0.25 — M_1–A
    - 0.8 — M_1–B
    - 1.0 — M_1–C
    - 1.2 — M_1–D
    - 1.4 M sucrose — M_1–E

b) scheme for osmotic shock procedure

SYNAPTIC VESICLES PREPARATION

The osmotic shock method was carried out according to DE ROBERTIS, *et al* (1963) *with some minor variations* *Osmotic shock* of fraction MIT was performed by suspending the pellet in 0 032 M sucrose containing 10 μM $CaCl_2$

Centrifuging at 10 000 g for 20 min eliminated nerve ending ghosts, myelin and osmotic shock resistant mitochondria indicated as fraction M_1 (Fig 1)

Fraction M_1 subjected to sucrose density gradient centrifuging could be split up in five fractions M_1–A–B–C–D–E which were approximately comparable with the fractions MIT–A–B–C–D–E The M_1 supernatant was centrifuged in the angle rotor at 105 000 g for 30 min and gave a sediment consisting largely of synaptic vesicles and membranes which after being resuspended in 0 25 M sucrose was called fraction M_2 Remaining hypo osmotic supernatant was called M_3

p Nitrophenylphosphatase activity was measured in an assay system with and without 3 mM Thiazinamium consisting of 4 mM *p* nitrophenylphosphate Na, 0 14 M Tris/HCl pH 7 2 and 0 7 mM $MgCl_2$

To 1 ml of this mixture 0 05 ml brain suspension was added and incubated at 37 °C for 60 min Reaction was stopped by 10 ml 0 02 M NaOH From the absorbancy at 415 nm the activity in μmol *p* nitrophenol/100 mg tissue wet weight × 60 min was *calculated with the aid of a standard curve* Thiazinamium was mainly used as representative of the phenothiazines because of its good solubility

RESULTS

The *p* nitrophenylphosphatase activity is considerably stimulated in homogenates of rat brain by phenothiazines at concentrations of 2 mM (DE WAART 1967)

A few other drugs we found also to be able to stimulate barbiturates however have no influence or are slightly inhibitory (DE WAART, unpublished) The stimulating effect with brain slices did not exceed 35 pCt *extra activity which is lower than that of* homogenate stimulation which exceeds 100 pCt Probably penetration into the particular cell area where the drug can exercise

Fig 1

homogenate

900 g for 10 min

NUC supernatant

10 000 g for 20 min

supernatant

105 000 g for 60 min

supernatant SUP MIC

crude mitochondrial fraction MIT pellet layered on discontinuous sucrose density gradient

130 000 g for 60 min

0 25 | 0 8 | 1 0 | 1 2 | 1 4 M sucrose

MIT-A | MIT-B | MIT-C | MIT-D | MIT-E

a) scheme for density gradient fractions

crude mitochondrial fraction MIT pellet resuspended in 0 032 M sucrose +10 μM $CaCl_2$

osm MIT

10 000 g for 20 min

supernatant

105 000 g for 30 min

supernatant M_3 M_2 pellet

M_1 pellet resuspended in 0 25 M sucrose layered on discontinuous sucrose density gradient

130 000 g for 60 min

0 25 | 0 8 | 1 0 | 1 2 | 1 4 M sucrose

M_1-A | M_1-B | M_1-C | M_1-D | M_1-E

b) scheme for osmotic shock procedure

its stimulating action is not undisturbed or unlimited. The stimulation in the slice preparations gave evidence that we have not measured an artefact as a result of damage to the cell structure

To get more information about the phenomenon of stimulation we subfractionated the homogenate. As we see from Table I the maximal localisation and stimulation is found in the crude mitochondrial fraction.

The microsomal fraction shows a high activity also, and is considerably stimulated, whereas the nuclear fraction and the supernatant have comparatively low activity and stimulation

TABLE 1

The influence of 3 mMol thiazinamium on p nitrophenylphosphatase activity [1]) in subcellular fractions of rat brain tissue

fraction	control = 100 pCt.	pCt stimulated activity
homogenate	5 80 ± 0 20	161
NUC	0 68 ± 0 08	124
MIT	1 70 ± 0 08	192
MIT-A	0 42 ± 0 02	132
MIT-B	0 29 ± 0 02	159
MIT-C	0 46 ± 0 03	196
MIT-D	0 25 ± 0 01	144
MIT-E	0 09 ± 0 01	100
osm MIT	2 14 ± 0 14	139
M_1	1 08 ± 0 05	135
M_1-A	0 22 ± 0 01	109
M_1-B	0 13 ± 0 01	125
M_1-C	0 15 ± 0 01	129
M_1-D	0 13 ± 0 01	119
M_1-E	0 01 ± 0 00	100
M_2 (synap ves)	0 45 ± 0 03	158
M_3	0 57 ± 0 08	114
MIC	1.44 ± 0 03	164
SUP	0 83 ± 0 04	131

1) Expressed in μ mole p nitrophenol formed per 100 mg dissected tissue in 60 min at 37 °C ± standard error of the mean

Figures were collected from five experiments

After density gradient centrifugation of fraction MIT, we can localize the highest phosphatase activity in nerve ending fraction C and the maximal stimulations in both nerve ending fractions (C and D) and in membrane fraction B High stimulation seems to be brought about by the presence of nerve endings, possibly not only in the crude mitochondrial fraction MIT, but also in the microsomal fraction, which surely *is contaminated with nerve* endings (WHITTAKER 1965) Various enzymic activities in a crude brain mitochondrial fraction are much higher if the fraction is resuspended in a hypo osmotic medium (SALGANICOFF and DE ROBERTIS 1965) In agreement with this observation, we have found a higher activity after osmotic shock of the crude mito chondria The resulting suspension called osm MIT and the fractions obtained therefrom by subfractionation again can be stimulated by Thiazinamium (Table 1)

Note that fraction M_2 consisting of synaptic vesicles and small membranes not sedimentable at 10 000 g shows the highest stimulation

When we compare activities and stimulations of the fractions MIT–A–B–C–D–E with those of the fractions M_1–A–B–C–D–E (Table 1) prepared by density gradient centrifugation of fraction M_1 it becomes clear, that the activity of fraction M_1–C is relatively diminished most and the stimulations of all fractions are much reduced as a result of the hypo-osmotic shock It seems that the strong stimulation of fractions B, C and C shifts after the shock to fraction M_2 and that for this reason the synaptic vesicles at least partly can be considered as the particles on which thiazinamium electively exerts a stimulating effect

DISCUSSION

AHMED and JUDAH (1964) and FUJITA *et al* (1966) described a *p* nitrophenylphosphatase, which parallels an ATP ase, and is stimulated by K^+ The efflux of K^+ from brain slices is reduced by chlorpromazine (MC ILWAIN 1962) One may account for the stimulation we observed by combining these data However there are some facts which are incompatible with this view First, if *p* nitrophenylphosphatase is stimulated by phenothiazines, why not Na^+–K^+– activated ATP ase (JARNEFELT, 1962, DAVIS and

BRODY, 1966) Secondly there is our finding, on which studies are in progress that glutamate decarboxylase, which is an enzyme not known to be stimulated by kations, is stimulated by thiazinamium likewise The fact that the stimulation is found in brain but hardly in liver or kidney (DE WAART, 1967) and that besides p nitro phenylphosphatase the typical brain enzyme glutamate decarboxyl ase present in the nerve ending axoplasma (SALGANICOFF and DE ROBERTIS, 1965) and in the mitochondria within the nerve endings (WHITTAKER, 1965), also is stimulated (DE WAART, 1967) indicates a mechanism which is a consequence of the unique structure of brain, *in concreto* the presence of the nerve endings in this tissue Indeed thiazinamium has a strong influence on the p nitrophenyl phosphatase activity in the typical nerve ending fractions MIT-C and –D Michaelis Menten constant measurements (DE WAART *et al*, 1967) of the stimulation phenomenon excluded a chemical interference between drug and the membrane bound phosphatase We suppose, as is postulated by ERNSTING *et al* (1960) and GUTH and SPIRTES (1964), that phenothiazines have the ability to affect membranes in general and in particular the permeability of mem branes In our case thiazinamium is able to alter the membrane permeability especially of the nerve endings, causing a higher p nitrophenylphosphatase activity We believe that this inter ference under the described conditions is so specific that this effect can be used as a tool for localisation of enzymic activity

From our experiments we conclude that the place of the stimu lating action can at least partly be localized in the synaptic vesicles within the nerve endings

The mode of action may be either a direct one on the membrane permeability of the vesicles possibly causing altered concentrations of transmitter substances within these storage particles or an indirect one by counteracting the tendency of the synaptic vesicles to clump, resulting in a relatively larger surface area and thus in a quicker equilibration of stationary concentrations

Although the stimulating action on the contents and membranes of the nerve endings is a suggestive phenomenon we find it too speculative to correlate it definitely with the *in vivo* influence of phenothiazines

SUMMARY

The crude mitochondrial fraction of rat brain was partly subjected to sucrose density gradient centrifugation and partly to osmotic shock treatment with subsequent sucrose density gradient centrifugation

p Nitrophenylphosphatase (p npp) activity of all these fractions with and without 3 mM thiazinamium (Multergan) was measured

Osmotically shocked mitochondria showed higher p npp activity than original crude mitochondria, but were considerably less stimulated by thiazinamium

In the sucrose gradient fractions of the crude mitochondria the thiazinamium stimulations were highest in the nerve-endings fractions

After osmotic shock treatment total activity as well as stimulation in the nerve-ending fractions were greatly diminished and a shift of stimulated activity to the synaptic vesicle fraction occurred

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3 4 2 *Miniature depolarizing potentials*

In Fig 12 the membrane potential is shown as recorded on a Goerz pen recorder (bandwidth 0–2 cycles per sec) Upon inspection of these minutes long tracks some of the phenomena mentioned before are seen For membrane potentials in the vicinity of zero the intensity of the noise is comparatively high Below about −70 mV a different behaviour is seen depolarizing peaks with quiet periods in between Intensity and duration of these phe nomena increase with increase of negative membrane potential

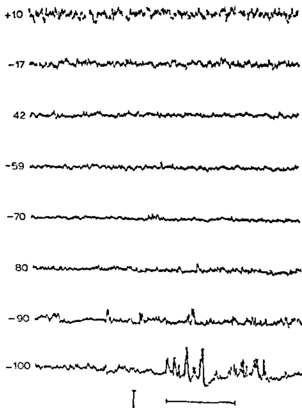


Fig 13
Membrane noise at different levels of membrane potential Figure com plementary to Fig 12 High pass filter with a time constant of 1 sec Units 5 mV and 1 sec Numbers membrane potential in mV The noise burst at the extreme right in the lowest track corresponds to the largest peak in the lowest track of Fig 12

In Fig 13 the actual noise tracks are shown as replayed from tape at low speed on a wide-band (0–100 Hz) pen recorder The intensity of the *noise increases with decrease of the negative* membrane potential

At −70 mV the level of the noise is minimal and an occasional *depolarizing peak may be present* Definite depolarizations of short duration are present at membrane potentials below −70 mV These depolarizations increase in amplitude and rate of occurrence with

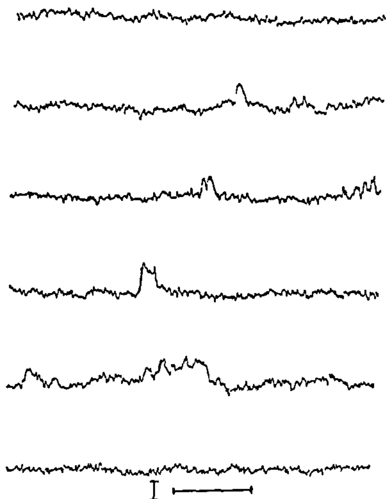


Fig 14

Membrane noise at a membrane potential of — 90 mV High pass filter with a time constant of 1 sec Units 1 mV and 10 msec The first and last tracks have Gaussian amplitude distributions A single miniature depolarizing potential is visible in the second track Clusters are present in tracks 3 through 5

increased negative membrane potential Negative undershoots are the consequence of the high pass filter used The high intensity noise in the right part of the lowest track of Fig 13 corresponds with the 20 mV depolarizing peak seen in Fig 12 In Fig 14 a selection of small depolarizing humps has been made from recordings at a magnified time base The recordings are from a node set at −90 mV The upper and lower tracks are Gaussian The second through fifth tracks show depolarizations of increasing complexity It is seen that the depolarizing phenomena are composed of additive unit phenomena miniature depolarizing potentials No answer can as yet be given about the precise amplitude and duration of the miniature depolarizing potentials, since the Gaussian noise level is too high From selected miniature depolarizing potentials our impression is for their amplitude to be of the order of 1 mV and their duration of the order of 1 msec (Fig 14, second track) with a triangular shape But the miniature depolarizing potential shown might be composed of two or more unit phenomena A complete two minute track at −90 mV and at a somewhat lower speed is presented in Fig 15 together with the actual track of the membrane potential on the recorder The miniature depolarizing potentials are seen to occur irregularly in groups of varying size and duration up to high intensity long duration noise bursts

3 4 3 *Role of ions*

Both the independence of the range of transition from the resting membrane potential and the shape and duration of the miniature depolarizing potentials suggest that the skewed noise is caused by a batchwise influx of sodium ions but that it can also be caused by batchwise chlorine ion influx and/or sudden decreases in potassium ion permeability

To investigate a possible relationship between skewed noise and ionic fluxes the following hypotheses were tested

1 Skewed noise is caused by active transport This hypothesis was rejected since blocking of active transport by 2,4-dinitrophenol in a nitrogen atmosphere did not change the noise pattern of the node, even after 100 min of intoxication, which caused a considerable decrease of the resting membrane potential

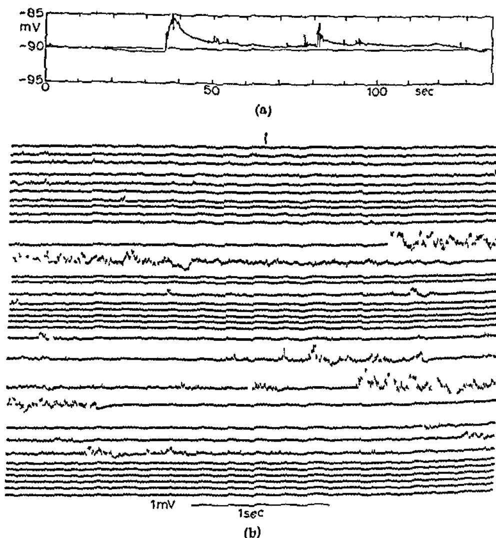


Fig 16

Membrane noise at a membrane potential of — 90 mV Two minute track replayed at low speed and a bandwidth of 0–2 cycles/sec (a) and at high speed and a bandwidth of 2–10000 cycles/sec (b), units for b 1 mV and 1 msec

2 Skewed noise is caused by changes in passive potassium membrane mechanisms Since both the membrane potential and the Gaussian noise minimum shift towards zero membrane potential in KCl-Ringer a similar shift is expected for the range of transition Such a shift was not found (Fig 9) The hypothesis is, therefore, rejected

3 Skewed *noise* is caused by passive chlorine ion fluxes In an experiment in which all chlorine ions in the Ringer's solution were replaced by sulphate ions, the concentration of all other substances remaining equal no change in skewed noise was found This hypothesis is therefore rejected

4 Of the major *membrane ionic mechanisms* all are eliminated except passive sodium fluxes A change in sodium ion concentration should therefore, cause changes in the skewed noise This has, indeed been found The relationship between the external sodium ion concentration and the position of the *transitional range is presented* in Figs 16 and 17 It follows that the lower the external sodium ion concentration is, the higher the negative membrane potential must be for the transition of Gaussian noise into skewed noise to take place

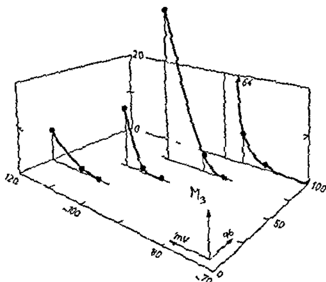


Fig 16
Relationship between *third moment* M_3 membrane potential *in mV* and sodium chloride concentration in *percentage* of that in Ringer's solution, for a single node of Ranvier *The level at which* the noise voltage changes from Gaussian into skew *shifts* towards higher levels of membrane *potential* (inside negative) when the concentration of *sodium chloride* in the solution decreases (*replacement* by saccharose)

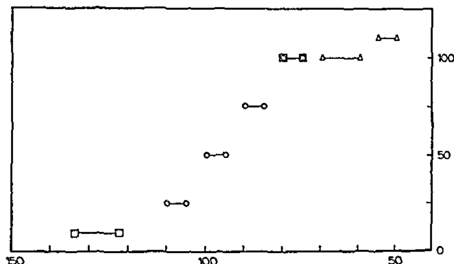


Fig 17
Range of transition for three nodes of Ranvier in different concentrations of sodium chloride Ordinate sodium chloride concentration in percentage of that in standard Ringer's solution Abscissa membrane potential in mV

It is concluded that positively skewed noise is caused by the passive batch-wise influx of sodium ions into the node or, more generally, that positively skewed noise is caused by spontaneous activation of passive sodium transport sites within the membrane

4 DISCUSSION

The existence of bursts of noise upon hyperpolarization of nerve-fibres has been mentioned in the literature In a study on the relationship of spontaneous miniature endplate potentials to polarization of the nerve endings DEL CASTILLO and KATZ (1967) made additional investigations on Ranvier nodes of the frog They described the existence of "breakdown effects" when the node is strongly hyperpolarized The asymmetry is clearly visible in their records (*cf* DEL CASTILLO and KATZ 1967 Figs 8 and 9)

They remarked that their recording conditions might have been unfavourable for the detection of quantal phenomena in these bursts Bursts composed of many unit potentials mask the units, however, independently of the recording conditions

DEL CASTILLO and KATZ ascribed the bursts to temporary breakdown of the membrane due to strong currents following a suggestion by HODGKIN (1947) But breakdown is excluded since

burst activity is present in nodes which exhibit high values of the negative resting membrane potential in Ringer in the absence of d c injection (Fig 11) When large amounts of current are injected into a node with a low resting membrane potential the node may become irreversibly damaged and such damage does occur pref erably during a noise burst However, other nodes can sustain strong currents for long periods without being destroyed In one case a node subjected to strong currents produced action potentials 24 h after the start of the experiment Positively skewed noise diminishes upon decrease of external sodium concentration It seems highly plausible to associate this phenomenon with an inflow of packets of sodium ions, occurring irregularly and in bursts

The question to be discussed here, is whether positively skewed noise can be caused by the spontaneous release of sodium ions from one or more individual sodium transport sites, each event leading to a small depolarizing impulse

ADOLPH (1963) showed that for a Poisson distribution of arrival times of exponential pulses of a shape e^{-t} highly asymmetric amplitude distributions result in the case of a low average rate of arrival per second For larger average rates the curves become increasingly symmetrical For large rates they approach the Gaussian distribution, as follows from the central limit theorem

Evidence for the existence of separate sodium channels has been given by CHANDLER and MEVES (1965) From results obtained with perfused squid giant axons they argue that the upper limit for the number of sodium sites would be 100 sites per square micron This amounts to an average number of 240 sodium ions which enter at one site during one action potential

Recently, MOORE, NARAHASHI and SHAW (1967) obtained a number of 13 sites per square micron, also for squid axon This number resulted from absorption measurements of tetrodotoxin on squid axon surfaces, the assumption being that each absorbed tetrodotoxin molecule blocks no more than one sodium channel and that no molecules are metabolized or absorbed on sites of a different kind

Accordingly, some 3400 ions would enter one site during the action potential Tentatively assuming that these quantities are valid for frog nodal membrane we find for a node of the following physical properties (TASAKI 1953 and TASAKI and FRANK, 1955)

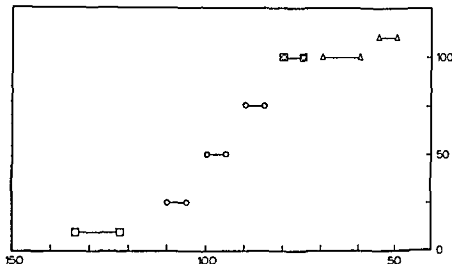


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One voltage jump ΔV due to discharge of one site is

$$\Delta V = \frac{\Delta Q}{C} = \frac{nq}{S C_s} \text{ Volts}$$

with $q = 1.6 \; 10^{-19}$ Coulombs

The signal to noise ratio

$$\frac{\Delta V}{(\overline{e^2})^{\frac{1}{2}}} = \frac{nq}{(2\pi r d C_s k T)^{\frac{1}{2}}}$$

will be larger for axons of smaller diameter r

For $T = 300$ °K, $C_s = 0.1$ Farad m^{-2}, $d = 1$ μm, $r = 2$ μm and with $n = 4000$

the voltage jump per site

$$\Delta V = 500 \mu V$$

and the ratio of the peak pulse amplitude to the r.m.s. of thermal noise

$$\frac{\Delta V}{(\overline{e^2})^{\frac{1}{2}}} = 8.2$$

The r.m.s. value of thermal noise is calculated as

$$(\overline{e^2})^{\frac{1}{2}} = 60 \; \mu V$$

while the experiments reported here and in DERKSEN (1965), and DERKSEN and VERVEEN (1966) indicate that the white noise level at the potassium equilibrium potential is of the order of 200 μV. The ratio of peak pulse amplitude to r.m.s. intensity of nodal noise voltage is about 2.5, i.e. much smaller than for thermal noise. For fibers of 2 to 4 μm diameter a reasonable chance of detection can, therefore, be expected. The use of a suitable preamplifier with a high input resistance, a very low input capacitance and a low noise figure for a source resistance from 50 to 100 MOhms is advisable.

SUMMARY

Membrane voltage noise of the frog Ranvier node has been investigated. In earlier investigations the structure of the noise spectrum has been reported. In the present report results are presented of investigations into the amplitude distribution

nodal diameter	r	4	μm
,, width	d	1	μm
,, surface area	S	12 6	μm^2
,, capacitance	C	1	pF

a number of sodium sites equalling 164, a maximum charge transfer of 8 0 10^{-14} Cb and an action potential amplitude of 80 mV, which is in the right order of magnitude

The hypothesis of single sodium transport sites leads to the expectation that the local responses caused by subthreshold stimulation of nerve fibre increase in discrete steps when the stimulus intensity is increased Such a phenomenon has been reported by DEL CASTILLO and SUCKLING (1957) and by LUTTGAU (1958)

The amplitudes of the unit potential changes of the local responses have been given as from 1 to 0 5 per cent of the action potential and as 0 6–1 1 mV respectively LÜTTGAU estimates that from 10^3 to 10^4 sodium ions enter through a single site This is again of the same order of magnitude

Detailed observation of individual discharges of sodium transport sites is hindered by the presence of Gaussian noise with a minimal intensity in the order of 200 μV *r m s* To increase the signal to noise ratio it would be advantageous to choose very small diameter myelinated axons

This can be shown as follows Given a node for which the membrane surface area $S = 2\pi rd\,\mu^2$, the nodal capacitance $C = S\ C_s$ Farads, with the specific capacitance C_s in F cm^{-2}, and let the average number of ions that pass per site during one discharge be n Thermal noise of an intensity

$$\overline{e^2} = 4\ k\ T\ R\ B\ \text{V}^2\ \text{sec}^{-1}$$

for a bandwidth

$$B = \frac{1}{RC}\ \text{cycles sec}^{-1}$$

gives

$$\overline{e^2} = \frac{kT}{2\pi r d C_s}\ V^2\ \text{sec}^{-1}$$

with $k = 1\ 6\ 10^{-23}$

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1 Between membrane potentials of zero and about 70mV (inside negative) the amplitude distributions are Gaussian at all levels of membrane potential
2 For membrane potentials larger than zero (inside positive) a tendency to negative skewness is present
3 For membrane potentials larger than about 70 mV (inside negative) the distributions are positively skewed Skewness increases with increase of membrane potential
4 Positive skewness is caused by the occurrence of noise bursts composed of clusters of miniature depolarizing potentials Intervals between bursts and size and duration of bursts are variable
5 Amplitude and duration of a miniature depolarizing potential are of the order of 1 mV and 1 msec respectively
6 In between noise bursts the amplitude distributions are Gaussian
7 The transition from Gaussian to positively skewed noise, measured with steps of 5 or 10 mV in membrane potential, differs for different nodes, with a mean value of about 70 mV and a dispersion from 60 to 90 mV (inside negative)
8 The level of transition is independent of the value of the resting membrane potential
9 The overall intensity of the membrane noise voltage is minimal at the transition from Gaussian to positively skewed noise
10 The intensity of Gaussian noise, measured for the range in which the noise voltage is Gaussian and for the Gaussian intervals when the noise voltage is skew, is minimal at the – supposed – level of the potassium equilibrium potential
11 Gaussian noise is related to the non metabolic flux of potassium ions through the membrane
12 Positively skewed noise is related to irregularly occurring inflow in batches of sodium ions through the membrane

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1 Between membrane potentials of zero and about 70mV (inside negative) the amplitude distributions are Gaussian at all levels of membrane potential
2 For membrane potentials larger than zero (inside positive) a tendency to negative skewness is present
3 For membrane potentials larger than about 70 mV (inside negative) the distributions are positively skewed Skewness increases with increase of membrane potential
4 Positive skewness is caused by the occurrence of noise bursts composed of clusters of miniature depolarizing potentials Intervals between bursts and size and duration of bursts are variable
5 Amplitude and duration of a miniature depolarizing potential are of the order of 1 mV and 1 msec respectively
6 In between noise bursts the amplitude distributions are Gaussian
7 The transition from Gaussian to positively skewed noise, measured with steps of 5 or 10 mV in membrane potential, differs for different nodes, with a mean value of about 70 mV and a dispersion from 60 to 90 mV (inside negative)
8 The level of transition is independent of the value of the resting membrane potential
9 The overall intensity of the membrane noise voltage is minimal at the transition from Gaussian to positively skewed noise
10 The intensity of Gaussian noise, measured for the range in which the noise voltage is Gaussian and for the Gaussian intervals when the noise voltage is skew, is minimal at the – supposed – level of the potassium equilibrium potential
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NETHERLANDS SOCIETY FOR PHYSIOLOGY AND PHARMACOLOGY

PROCEEDINGS NINTH FEDERATION MEETING MEDICAL-BIOLOGICAL SOCIETIES, NIJMEGEN, APRIL 18–19, 1968

F W Bauer *Isolation of polysomes from rat epidermis*

Department of Dermatology St Radboud Hospital Nijmegen

In the mammalian epidermis there is primarily one protein that is synthetized keratin Therefore the skin is a suitable object for the study of the protein synthesis in a cell free system

FREEDBERG *et al* (1967) described a system isolated from mice and guinea pig skin which exhibits a slight incorporation of exo genous amino acids into protein The authors however were able to isolate ribosomes but not intact polysomes Since as is generally accepted protein synthesis takes place on polysomes the system employed by these workers is probably not the most favourable for the study of keratin synthesis

Starting with pure epidermis separated from the dermis by incubating the skin of one-day-old rats in 2 25 % trypsin solution for 45 min at 37 °C allows subsequent isolation of a substantial polysome fraction in addition to ribosomes In the present research isolation was effected via sucrose gradient centrifugation and the particles were identified in electron microphotographs of the different fractions

REFERENCE

FREEDBERG I M I H FINE and F H CORDELLE J Invest Derm 48 55 (1967)

G J Barendsen L de Pater and Jw van den Berg *Pulse wave velocity in human arm arteries as a function of transmural pressure and respiration*

Laboratory of Medical Physics University of Groningen

Elastic characteristics of arteries can be studied by measuring the pulse wave velocity (or the propagation time) considering that the modulus of elasticity of the arterial wall material varies with the transmural pressure

In this study the propagation time in human arm arteries was measured over a range of transmural pressures between 30 % and 1‾0 % of the normal diastolic transmural pressure The transmural pressure was changed by applying external pressures between −60 cm H_2O and +80 cm H_2O to the arm

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Department of Physiology, University of Leiden

AMPLITUDE DISTRIBUTION OF AXON MEMBRANE NOISE VOLTAGE *)

BY

A. A VERVEEN AND H E DERKSEN

1 INTRODUCTION

It was reported earlier (VERVEEN and DERKSEN, 1965, DERKSEN, 1965, DERKSEN and VERVEEN, 1966) that the resting potential of the frog Ranvier node shows fluctuations with a spectral intensity N characterized by $N = K_1 + (K_2/f)$ in the frequency range 1–10 000 radians per second The first term, K_1 signifies so-called "white noise" and has been found to be somewhat larger than the thermal

*) This work was supported by grant no 761-14 from the Netherlands Organization for the Advancement of Pure Research (Z W O)

The propagation time was deduced from the time between the R-top of the E.C.G. and the lowest point of the photoplethysmogram of the finger. Corrections were made for the time lag between the R-top of the E.C.G. and the opening of the heart valves and for that part of the arterial tract that is not affected by the external pressure.

The propagation time was converted to a proportional d.c. voltage and continuously recorded together with the external pressure, the photoplethysmogram and the respiratory movements of the chest wall, the latter being measured by a strain gauge.

Twelve healthy subjects were studied. With decreasing transmural pressure to 30 % of the normal diastolic transmural pressure the mean propagation time increased nearly exponentially to 250 % (S.D. 20 %). With increasing transmural pressure above 120 % the propagation time was nearly constant, probably as a result of the practically indistensible sheath enclosing the artery with its venae comitantes.

The relation of the propagation time and the respiratory movements was studied by measuring the propagation time in each phase of the respiration cycle. The propagation time varied 6 % to 10 % synchronously with the respiration at normal transmural pressures, which implies, according to the afore-mentioned experiments, diastolic blood pressure variations of about 10 mm Hg, in agreement with the values found in the literature.

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E. J. Becker and F. Kreuzer, *Recommended "normal" values for urinary catecholamine excretion by healthy adults*

Department of Physiology, University of Nijmegen

Modern techniques for the determination of adrenaline and noradrenaline make the quantitative study of sympathoadrenal function in health and disease more meaningful. By measurement

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external shunting resistances along the myelin sheaths (DERKSEN, 1965) The pools were connected to low noise, chopper stabilized electrometer amplifiers (DERKSEN, 1965) and with a d c injection system This was done with KCl agar bridges to Ag–AgCl electrodes for the middle node – the node to be investigated – and with Ag–AgCl electrodes in the other pools

The Ag–AgCl electrodes were made from 2 mm diameter pure silver wire, electrolytically plated with AgCl Only sets of electrodes with interelectrode potential differences less than 2 mV were used For most electrodes these potential differences were of the order of 0 5 mV

For diagrams of set up and circuity we refer to DERKSEN (these Acta, 1965 pp 391–427) The outputs of the two main amplifiers were displayed on an oscilloscope The membrane potential of the middle node, measured *via* the branch through which no current was injected, was recorded on a Goertz Servogor recorder (bandwidth 0–1 cycles/sec) and on one track of an Ampex FR 1300 f m. tape recorder The same signal was fed through a high pass filter with a time constant of 2 seconds to eliminate the d c component – the (mean) membrane potential – and after additional amplification the membrane noise voltage was recorded simultaneously on another track of the tape

Analyses of membrane noise were made for different nodes and for each node at different levels of membrane potential, the levels being set by injection of direct current For each node the analysis was made both with Ringer and with one or more modified solutions

In all measurements a small part of the noise represents amplifier noise (about 5 per cent of the noise level recorded at minimal intensity of membrane noise) Since this noise is independent of nodal membrane potential and does not obscure our measurements (DERKSEN, 1965), no corrections for amplifier noise have been made

All experiments were made at room temperature

3 RESULTS

3 1 AMPLITUDE DISTRIBUTIONS

3 1 1 *Characteristics*

The voltage noise signal was sampled with pulses with a width

noise intensity for the membrane The 1/*f* term, *i e* the noise component for which the power per cycle of bandwidth is inversely proportional to frequency, is related to the passive flux of potassium ions through the membrane (DERKSEN, 1965, DERKSEN and VERVEEN, 1966)

For frequencies down from 1 rad/sec an increase in negative slope of the log noise power *vs* log frequency line may occur (DERKSEN, 1965) which was found to be associated with the occurrence of small depolarizing transients of the membrane potential

The present paper is a report on an investigation of the amplitude probability density distributions of the membrane noise voltage A preliminary report was published earlier (VERVEEN, DERKSEN and SCHICK, 1967)

2 METHODS

Isolated nerve fibers from the sciatic nerve of the frog *Rana temporaria* were used Each fiber was prepared over a length of three nodes, care being taken to clean the fiber as much as possible from tissue remnants The nerve fiber was mounted in a chamber with open perfusion, made of the polycarbonate Makrolon (Bayer AG) The central part of the chamber [1]) consisted of five pools (*cf* DERKSEN, 1965, p 412), over which the nerve fiber was laid with the middle node in the central pool, through which the testing fluid flowed, and with each of the other nodes in one of the outer pools To depolarize the outer nodes their pools were filled with distilled water, which was replaced by isotonic KCl solution The intermediate pools, each in contact with part of each internode, were filled with Ringer solution and used for the electronic feedback isolation (DERKSEN, 1965) developed after FRANKENHAEUSER (1957)

The parts of the internodes lying on the ridges between the pools were carefully covered with silicone grease [2]) both to prevent drying of these segments and to provide maximal values for the

[1]) A detailed description of the preparation techniques and of the chamber and perfusion system, which have been redevised, will be published separately

[2]) Dow Corning high vacuum silicone grease (Dow Corning Corporation, Midland, Michigan, U S A) was used instead of petroleum jelly, following a suggestion made by Mr J Bouman

external shunting resistances along the myelin sheaths (DERKSEN, 1965) The pools were connected to low noise, chopper-stabilized electrometer amplifiers (DERKSEN, 1965) and with a d c injection system This was done with KCl agar bridges to Ag-AgCl electrodes for the middle node – the node to be investigated – and with Ag-AgCl electrodes in the other pools

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3 RESULTS

3 1 AMPLITUDE DISTRIBUTIONS

3 1 1 *Characteristics*

The voltage noise signal was sampled with pulses with a width

of 1 5 μsec at a frequency of 1000 pulses per second The amplitude (x) of each sample was stored in the memory of a 128 channel RCL iac scaler analyzer with a bin width of 0 1 mV of the original signal (Fig 1) After exactly 100 000 samples had been taken the frequency distribution of the amplitudes was read out

In all cases the amplitude distributions were found to be uni modal The number of classes varied between about 15 and about 60 per distribution The cumulative amplitude distributions were plotted on Gaussian paper (*cf* Fig 9b) All non Gaussian distri butions were found to be skewed The few distributions for which graphical classification was difficult are not of importance with respect to the problems investigated All distributions were analysed graphically by plots made on semilog paper, on which Gaussian

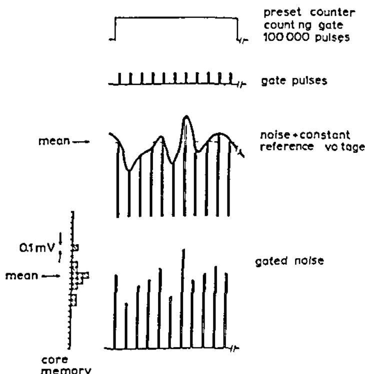


Fig 1
Determination of the amplitude distribution of noise voltage
Gate pulses are counted by a preset counter for a total of 100 000 pulses For each gate pulse the amplitude of the noise is measured and one unit is added to the corresponding amplitude bin in the core memory of a scaler analyser Since the *mean noise voltage is zero* a *constant reference voltage* is added to the noise before gating

distribution functions are parabolas, while disturbances in the tails are easily noted (cf. Fig. 5a). Measures M_n for the moments of the distributions were graphically determined. In some cases the moments $\mu_n = (\Sigma x^n / N)$ with $n = 1, 2, 3$ and $N = 100\,000$ were also computed with the aid of an IBM 7090 digital computer [1]) to check the graphical procedures.

3.1.2 *The first moment*

The first moment is the mean value of the membrane potential. For the distributions measured the mean is equal to zero because of the use of a high pass filter. A graphical estimate of the mean value was, however, made in order to determine the higher moments. A line is drawn through the 100-count level of the distributions. A perpendicular line through the maximum indicates the mean (Fig. 2). All means were found to lie between +0.2 and −0.2 mV, even for skewed distributions, while the differences between computed and graphically determined values were small (14 values: mean difference 0.01 mV, largest difference 0.08 mV).

3.1.3 *The second moment*

For the graphical determination of measures for dispersion and skewness the two segments a and b (Fig. 2) were read off in units of 0.1 mV with an accuracy of about 0.02 mV. The sum $s = a + b$ is used as an estimate of dispersion. In Fig. 3 the estimate $M_2 = s^2$ is plotted *vs* the mean μ_2 as computed for series containing both Gaussian and non Gaussian (s larger than 7) distributions. It follows that M_2 is a reliable measure for the second moment μ_2 (the variance or power). In the regression line $M_2 = \alpha + \beta\mu_2$, $\alpha = -0.13 \pm 0.18$ [2]) and $\beta = 42 \pm 0.92$. The corresponding relationship between the sum s and the standard deviation (r.m.s. value) $\sigma = \sqrt{\mu_2}$, taking $\alpha = 0$, is $s = 6.5\,\sigma$. For the experiments to be mentioned plots of s *vs* membrane potential are presented with s expressed in units of 0.1 mV. The r.m.s. value σ can, therefore, be read from the graphs after division of s by 6.5. The method is an approximation

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[1]) For $n = 2$ and $n = 3$ the moments around the mean were calculated. Thanks are due to Mr. J. Roukens who wrote and executed the programmes.

[2]) Two times the standard deviation. 14 points.

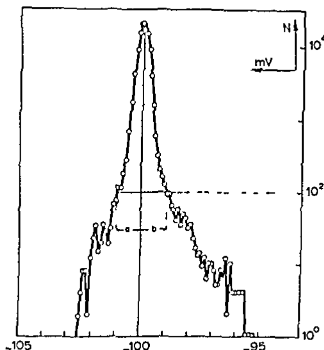


Fig 2
Graphical procedure used for the estimation of measures for the first three moments of an amplitude distribution
abscissa membrane potential in mV,
ordinate counts per bin
For explanation see text

of the determination of the 0 1 to 99 9 percentile range For the percentile interval between $P(x) = 0\ 001$ and $0\ 999$, $s = 6\ 2\sigma$ At a probability density $p(x) = 0\ 001$ the cumulative probability $P(x)$ is in most cases smaller than 0 001 and larger than 0 999 respectively, so a value for s somewhat larger than 6 2 is to be expected

The level $p(x) = 0\ 001$ is chosen because it corresponds with the 100 count level and is easy to read off For lower levels the distribution loses its smoothness, for higher levels the distances a and b become too small and the relative error in measuring these distances becomes too large

3 1 4 *The third moment*

The graphical measure M_3 for the third moment or mean cube value μ_3 is used as an indicator of skewness Since M_3 can be either positive or negative and in both cases either large or small

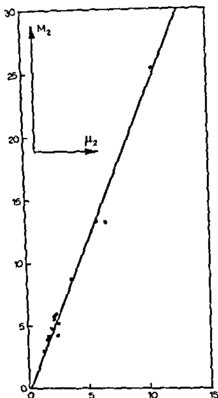


Fig 3
Computed second moments μ_2 plotted *vs* graphically determined measures M_2 of 14 amplitude distributions For scale units see text.

an *arc tan* representation is used for plots of M_3 *vs* membrane potential This representation is bounded at $+\pi/2$ and $-\pi/2$ at the limits for M_3 equal to $+\infty$ and $-\infty$ respectively The graphical estimate M_3 for the third moment μ_3 used here is the cubed difference between b and a $M_3=(b-a)^3$ and is expressed in units of (0 1 mV)³ Since the range of unit values of M_3 found here is between 0 and ± 1000 the plots are given for *arc tan* $\frac{1}{10}M_3$ to [illegible] the graphs In Fig 4 a plot of [illegible] ven for series of both Gaussian [illegible] istributions It follows that M_3 is a reliable measure of μ_3, but with a loss of sensitivity for $|M_3|$ smaller than about 2 units A coefficient of skewness, such as $\mu_3/\mu_2^{3/2}$

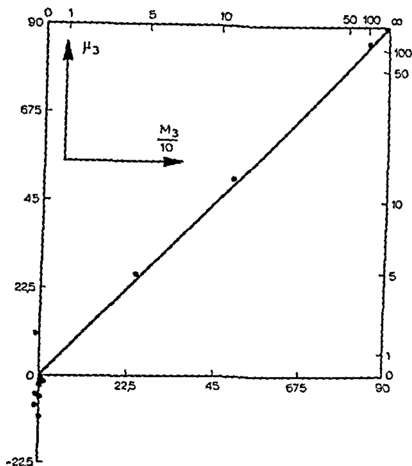


Fig 4
Arc tan plot of third moments μ_3 vs graphically determined measures M_3 of 14 amplitude distributions Above and right scale units (see text), below and left degrees of arc

was not used in this series of experiments, since the results to be presented do not call for more detail as yet, while the asymmetry has been reduced by passage through a high pass filter (cf section 2 1) The distribution will not become symmetric, however, but the skewness is reduced by this procedure The measures M_3 were mainly used to determine the level of membrane potential at which a change from Gaussian to skewed distributions did occur

3 2 Shape and properties of amplitude distributions

The amplitude distributions of membrane voltage noise exhibit a typical relationship to membrane potential An example is given

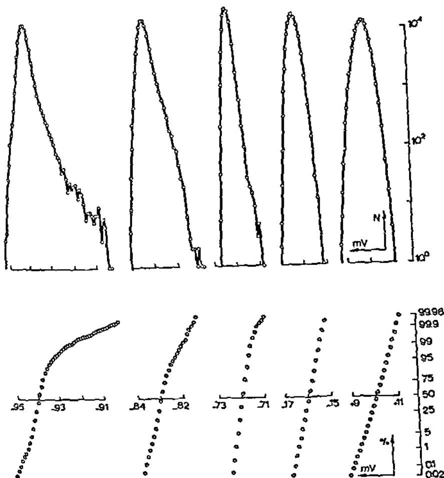


Fig 5
Amplitude distributions of a frog node of Ranvier for different values of the membrane potential (abscissae) Top semilog plot of the cumulative distributions, counts per bin Bottom Gaussian plot of the cumulative distributions, ordinate percentage of total number of samples

in the semilog plot of Fig 5a In Fig 5b the same distributions are shown, plotted as cumulative distributions on Gaussian paper The distributions are Gaussian for membrane potentials between about −75 mV and 0 mV. For membrane potentials of about zero a slight negative skewness is present, a phenomenon which has not

been further investigated as yet Below about −70 mV the distributions exhibit a positive skewness, an asymmetry towards a less negative potential. This is visible as a positive tail, the righthand tail in the plots When these tails are pronounced, a smaller tail is seen at the left This tail is due to the effect of the high pass filter and has no physiological implications Said relationship between amplitude distribution and membrane potential has been found in all nodes investigated, irrespective of the actual value of the membrane potential recorded in the absence of injection of d c current

The relationship between the measure *s* of the *r m s* value of membrane noise voltage and membrane potential is depicted in Fig 6

For membrane potentials between zero and −70 mV M_2 decreases with increase of membrane potential The dispersion of these values is small

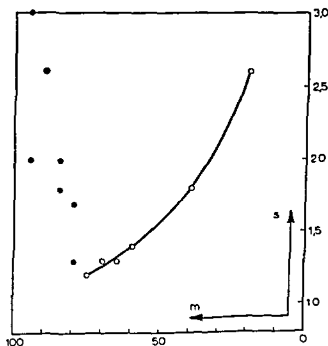


Fig 6

Plot of the measure for noise intensity *s* (mV) *vs* mean membrane potential *m* (mV) for a node of Ranvier Length of noise tracks analysed per point 100 sec A line has been drawn by eye through the points corresponding with Gaussian distributions (open symbols) Filled symbols positively skewed distributions

For membrane potentials more negative than about −70 mV s increases sharply, while the dispersion of s measured for several different samplings at the same potential is large For the Gaussian range different samples at the same potential have similar values of s A closer investigation was made by sampling in blocks of 100 000 at a rate of 8000 samples per second Within the Gaussian range 3 samples were taken at each level of membrane potential and within the skewed range 9 samples per level were taken The resulting values for s are presented in Fig 7 Even in the range of negative potentials down from −70 mV a few Gaussian distributions (open circles) are found A line drawn by eye through the open circles has a definite minimum, in this case at about −80 mV Within the range for skewed noise both Gaussian and non Gaussian sequences occur

3 3 Gaussian noise

The Gaussian amplitude distributions within the range of membrane potentials between about zero and about −70 mV, and the Gaussian distributions interspersed among the non-Gaussian distributions for membrane potentials in excess of about 70 mV, inside negative, exhibit a minimum intensity at membrane potentials of about −80 mV or more, i e in the vicinity of the potassium equilibrium potential

It was shown earlier (Derksen, 1965, Derksen and Verveen, 1966) that $1/f$ noise is of minimal intensity at the level of the potassium equilibrium potential and is related to the passive flux of potassium ions through the membrane One might ask, therefore, whether the Gaussian noise as found in the investigation of the amplitude distributions of membrane voltage noise is also related to potassium ion flow

To investigate a possible relationship between Gaussian noise and the transport of ions the following hypotheses were tested

1 Gaussian noise is related to active transport Blocking of active transport should, therefore, reduce its overall intensity In an experiment with 2,4-dinitrophenol in a nitrogen atmosphere no influence on the intensity of the noise was found Active transport of ions is, therefore, excluded

2 Gaussian noise is related to Cl′ and/or Na flux A change in

been further investigated as yet Below about −70 mV the distributions exhibit a positive skewness, an asymmetry towards a less negative potential This is visible as a positive tail, the righthand tail in the plots When these tails are pronounced a smaller tail is seen at the left This tail is due to the effect of the high pass filter and has no physiological implications Said relationship between amplitude distribution and membrane potential has been found in all nodes investigated irrespective of the actual value of *the membrane potential recorded in the absence of injection of* d c current

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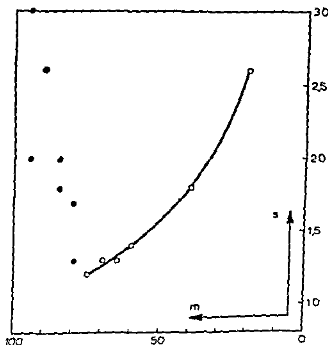


Fig 6

Plot of the measure for noise intensity *s* (mV) *vs* mean membrane potential *m* (mV) for a node of Ranvier Length of noise tracks analysed per point 100 sec A line has been drawn by eye through the points corresponding with Gaussian distributions (open symbols) *Filled symbols* positively skewed distributions

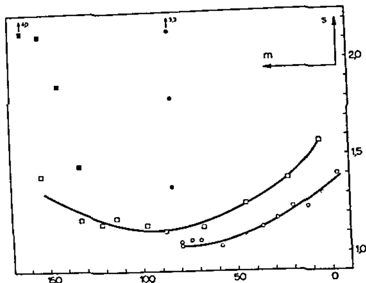


Fig 8

s — m plots for a single node of Ranvier in Ringer's solution (circles) and in 10 per cent NaCl Ringer (squares) Cf Fig 6

s
m
3,0
2,5
2,0
1,5
1,0
100
50
0
+50

Fig 9

s — m plots for two nodes of Ranvier In Ringer's solution (circles) and in KCl Ringer (squares) for one node In KCl Ringer (triangles) for another node Arrows indicate the resting membrane potential in KCl Ringer

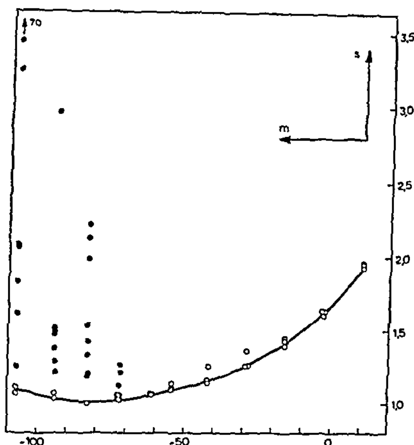


Fig 7
s—m plot of another node Length of noise tracks analysed per point 12 5 sec *Cf* Fig 6

external Cl′ concentration and/or Na concentration should *then change the position of the level of minimal intensity* *This* was tested in an *experiment in which the* NaCl concentration of the Ringer solution was reduced to 10 per cent of the original concentration by replacement of NaCl by saccharose (Fig 8) The minimum intensity of the Gaussian noise was found to *remain in the vicinity* of the potassium equilibrium potential Both Cl′ and Na are, therefore, excluded

3 From the results mentioned under (1) and (2) it follows that the passive flux of potassium ions through the membrane remains as a possible cause for the Gaussian noise

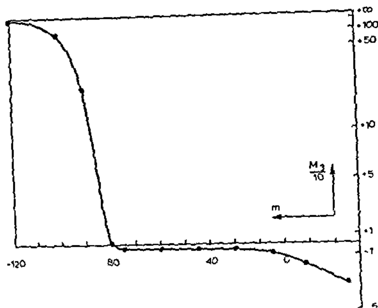


Fig 10
Third moment (its measure M_3, for units see text) *vs* mean membrane potential *m* (in mV) for a node of Ranvier For explanation see text.

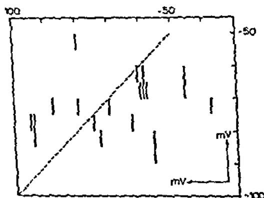


Fig 11
Range of transition (ordinate in mV) *vs resting* membrane potential (abscissa in mV) as found for the nodes investigated *Nodes for* which the range of transition lies to the left of the 45° line through the origin (*dashes*) show noise bursts in the resting state.

Results of experiments with KCl-Ringer (replacement of NaCl by KCl, other ions unchanged) are shown in Fig. 9. The minimum shifts toward zero membrane potential. For one node the minimum is seen exactly to coincide with the resting membrane potential. The other node is even more interesting, since for this fiber the minimum is at -11 mV *i.e.* between the resting membrane potential of -21 mV and zero. In this case the contribution of chlorine ion concentration to the membrane potential is, therefore, not negligible (STÄMPFLI, 1959). It follows that in all probability the minimal level of Gaussian noise exactly indicates the value of the potassium equilibrium potential. Since we could not measure the internal potassium concentration of the node this could not be tested in a direct experiment.

From these experiments it is concluded that the intensity of Gaussian noise of frog nodal membrane is related to the net passive flux of potassium ions through the membrane.

3.4. Positively skewed noise

3.4.1. *Amplitude distribution*

In Fig. 10 a plot of the measure for skewness M_3 *vs* membrane potential is given.

Negatively skewed noise is found for levels of membrane potential in the vicinity of zero, increasing with increase of inside positivity of the node, but has not been investigated further as yet.

A comparatively sharp transition from Gaussian noise into (positively) skewed noise occurs at membrane potential levels of about -70 mV. Since the samples were taken after changes of the membrane potential in steps of 5 or 10 mV the range of membrane potential in which transition of Gaussian noise into skewed noise occurs is registered.

For 15 nodes the transitional ranges are presented in Fig. 11, plotted against the resting membrane potential (measured in the absence of d.c. injection). Nodes with values of the membrane potential which are more negative than their range of transition spontaneously exhibit skewed noise, which changes into Gaussian noise upon sufficient depolarization, while the noise of the other

of plasma catecholamine levels rapid, transient changes can be followed, but because of the extremely low concentrations of free A and NA in normal blood the accuracy of blood catecholamine determinations is limited Also, large volumes of blood are needed and in many cases the anxiety involved in obtaining the blood sample may give higher values

Normal urine contains about 25 times more free A and NA than blood and the determinations are much more accurate than for plasma Collection of urine specimens involves no stress factors and collection can be repeated It is true that only a very small fraction of the total active amines produced by the sympatho adrenal system appears in the urine in unchanged form, yet there seems to be a close relationship between the amount secreted into the blood and the amount excreted in the urine For these reasons urinary catecholamine determination is currently regarded as the best method for the assessment of the overall sympathoadrenal activity (VON EULER, 1964)

Hydroxymethoxymandelic acid is the major metabolic break down product of both NA and A Since 60 to 80 % of the free amines produced will be excreted in this form (DAUCHY and GIUDICELLI, 1965), determination of HMMA in the urine can give information about the total amount of active catecholamines produced during a given period of time

For studies of long duration the 24 h excretion figures are usually satisfactory Analysis of separate urine samples collected during shorter periods of time can give information about changes in sympathoadrenal activity owing to stress conditions operating only for shorter time periods

Obviously, for all quantitative experiments the normal excretion values are needed as a basis for comparison No such values are at present generally accepted The present study was designed to obtain normal excretion values by determining the adrenaline, noradrenaline, and hydroxymethoxymandelic acid excretion of 27 healthy adults of both sexes under everyday conditions

The following average 24 hour excretion values were found for men 24 3 μg NA 7 1 μg A, 6 0 mg HMMA and for women 23 1 μg NA, 5 6 μg A, 6 2 mg HMMA

The well known diurnal variation of catecholamine excretion should be taken into consideration when results are compared from

nodes is Gaussian and becomes skewed upon hyperpolarization The most frequently occurring range of transition is from −70 to −75 mV, with extremes of − 50 to −55 mV and −85 to −90 mV. It follows from the graph that the position of the transitional range does not depend on the actual value of the resting membrane potential recorded in the absence of d.c. injection. This observation suggests that skewed noise is not related to mechanisms that maintain the resting membrane potential.

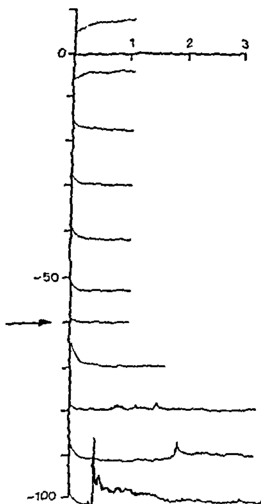


Fig. 12

Time course of membrane potential at different m p values Low-pass filter with time constant of 1 sec. DC. injection Ordinate values in mV, abscissa values in minutes Arrow: resting membrane potential of this node Note the depolarizing nature of the noise bursts

corded. DDT causes a prolongation of the action potential and an increase of the negative afterpotential DDT has no effect on the resting potential

We acknowledge the help of the Shell Cy in this investigation

REFERENCE

STÄMPFLI R, Ann New York Acad Sc 81, 265 (1959)

B Bink and F Wafelbakker, ***Physical working capacity of boys 12–18 years of age***

Netherlands Institute for Preventive Medicine, Leiden

The question about (maximum) physical working capacity of adolescents has been raised in three fields occupational medicine, labour legislation and sport medicine

In The Netherlands, in contrast to other countries, only little ergometric research in adolescents has been done Measuring the maximum working capacity of young people is an item that has hardly been covered elsewhere

In 51 boys from 12 to 18 years old the maximum physical working capacity was measured by means of a bicycle ergometer with a continuously growing load Oxygen consumption was assessed by the 'open method", using Douglas bags and Haldane gas analysis

The highest performance of boys was found to increase from round 150 Watts for twelve year olds to round 250 Watts for eighteen year olds

The maximum oxygen intake increased, at the same time, from 1 9 liter/min to 3 4 liter/min Per kg of body weight, the maximum oxygen intake was constant, at a mean of 51 ml

The Physical Working Capacity at heartrate 170 (PWC 170) doubled between 12 and 18 years from 109 to 201 Watts

The Pulse Capacity Index (PCI) defined as the increase in pulse rate per rise of 10 Watts in the external load, diminished from 7 in twelve year olds to 4 in eighteen year olds, which is the normal value for adult men

The pulse capacity index was found to be the only index that correlates to practising sports, as follows from the table

samples collected during different parts of the day The subjects of the present study excreted on the average about 20 % less total free catecholamines during the afternoon than during the morning and about 60 % less during the night than during the morning

It is becoming customary to express catecholamine excretions either as nanograms per minute or as nanograms per milligram creatinine Since the average adult human excretes about 1500 milligrams of creatinine per day, the two ways of expression give essentially identical values

Detailed results of this study will be reported elsewhere

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J van den Bercken and L. Akkermans, *The effect of DDT on single nerve and muscle fibres of Xenopus laevis*

Institute of Veterinary Pharmacology and Toxicology University of Utrecht

By recording action potentials of single myelinated nerve fibres by means of a suction pipette electrode it has been shown that DDT causes a number of fibres to discharge repetitively to a single shock

A more detailed analysis of the effect of DDT was made on isolated nodes of Ranvier (STAMPFLI 1959) In some fibres DDT (5×10^{-4} M) causes a prolongation of the falling phase of the action potential In other fibres this prolongation during the first part of exposure is less clear In these fibres DDT causes a long large negative after potential No noticeable change was observed in the resting potential level In these isolated nodes repetitive action potentials after treatment with DDT were not recorded Since we very probably isolated only motor nerve fibres this may indicate that the DDT induced repetitive action potentials appear only in sensory nerve fibres

The effect of DDT on striated muscle fibres was studied by means of intracellular capillary microelectrodes After intraperitoneal injection of DDT (25 mg/kg b w) resting and action potentials of the isolated curarized semitendinous muscle were re

corded DDT causes a prolongation of the action potential and an increase of the negative afterpotential DDT has no effect on the resting potential

We acknowledge the help of the Shell Cy in this investigation

REFERENCE

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The Physical Working Capacity at heartrate 170 (PWC 170) doubled between 12 and 18 years from 100 to 201 Watts

The Pulse Capacity Index (PCI), defined as the increase in pulse rate per rise of 10 Watts in the external load, diminished from 7 in twelve year olds to 4 in eighteen year olds which is the normal value for adult men

The pulse capacity index was found to be the *only index that* correlates to practising sports as follows from the table

TABLE I

51 boys Leiden Technical School 12–18 years

age (years)	n	weight (kg) mean	max effort (Watt) mean	max V_{O_2} (l/min) mean	max V_{O_2}/kg (ml/min) mean	PWC 170 (Watt) mean	PWC 170/kg (Watt) mean	PCI mean	PCI member sporting club	
									yes	no
12	8	38 6	155	1 96	50 9	108 9	2 8	7 27	6 6	8 4
13	9	44 1	165	2 22	51 0	126 0	2 9	5 96	5 8	6 2
14	7	49 4	198	2 51	50 9	149 0	3 0	5 72	5 3	6 3
15	7	54 7	210	2 73	50 1	178 6	3 2	4 73	4 6	5 2
16	13	59 8	238	3 23	54 4	177 4	3 0	4 91	4 9	4 9
17	5	63 2	246	3 07	48 2	182 2	2 9	4 45	3 8	4 9
18	2	64 0	255	3 42	53 4	201 5	3 2	4 19	—	4 2

J L Blom, *Quantitative analysis of firing patterns of cortical neurons*

Netherlands Central Institute for Brain Research, Amsterdam

"Spontaneous" electrical activity of single neurons in the rabbit sensory motor cortex was recorded with micro-electrodes under steady state conditions Extracellularly recorded action potentials were sampled on magnetic tape for analysis

a Mean firing frequency During a 10 minutes period the mean firing frequency of the neuron was determined The overall frequency for 318 neurons was determined to be 5 23 (S E 0 29) action potentials per second A significant increase in firing frequency was found from 400 μ below pial surface onto the white matter

b For 24 neurons an autocorrelogram was made of 400 consecutive time intervals Only 8 neurons showed a significant correlation for the first order correlation coefficient at the 1 % level It was concluded that the first order interval analysis did not show a dependency, thus the interval histogram may be analysed by stochastic statistical techniques

c Of all neurons (318) interval histograms were made with the aid of a data retrieval computer (Nuclear Chicago 7100) The results were plotted on graphic paper and also punched on paper tape for further computer analysis

The results will be published in extenso in the near future

J Th F Boeles, H Beuningh and G J Lankhorst, *Spontaneous mechanical activity of perfused segments and isolated arteries of the human uterus*

Department of Physiology and Department of Obstetrics and Gynecology University of Amsterdam

Isolated segments of human gravid and non gravid uteri were perfused with Tyrode solution by means of a constant volume pump Both the pressure fluctuations in the perfused uterine artery and the contractions of the segment were recorded

Oxytocin vasopressin, adrenaline, noradrenaline and ergonovine simultaneously increased the perfusion pressure and the spontaneous mechanical activity of the gravid and non-gravid myometrium Occasionally, however, the rhythmic fluctuations of the perfusion

pressure and the spontaneous myometrial contractions were not synchronous. Moreover, the amplitude of the pressure variations and the height of the uterine contractions were not always closely related to each other.

On these grounds we assumed that sometimes another factor besides myometrial motility might alter the resistance of the uterine vascular bed. For this reason we measured isometrically the tension of transverse, 4–6 mm long strips taken from isolated arteries of both gravid and non-gravid human uteri. The strips were immersed in Tyrode solution at 37 °C and gradually stretched till normal wall tension was reached. After 2–3 h of incubation spontaneous rhythmic contractions appeared, which continued for hours. The frequency of the regular, strong contractions varied between 10–24/h, whereas small, irregular contractions of a much higher frequency were sometimes superimposed on the former.

Vasopressin, adrenaline, noradrenaline and ergonovine caused a rise of tone and sometimes evoked spontaneous activity, whereas *oxytocin always decreased arterial wall tension in both gravid and* non-gravid uteri.

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SOMLYO, A. V., C. Y. WOO and others. Am. J. Physiol. 208. 748 (1965).

F. H. Bonjer, *Comparability of exercise tests carried out in different centres*

Netherlands Institute for Preventive Medicine, Leiden

If the aerobic capacity of samples of the population all over the world is to be assessed in order to study the influences of race, climate, nutrition and habitual physical activity, there should be a guarantee that the results of exercise tests are really comparable.

World-wide comparability requires more than national agreement. With this in mind six centres in The Netherlands agreed to set up a national study, which can be considered as a preliminary study for the International Biological Program.

Laboratories for physiology belonging to the Universities of Amsterdam, Utrecht and Nijmegen, the medical service to the Netherlands Railways and the institute for chest diseases of the

Coal Mines co operated with the department for Occupational Medicine of the Institute for Preventive Medicine

Twelve Olympic cyclists served as subjects exercising twice at each of the six centres in a random order The tests were carried out according to a scheme of stepwise increase of the load Usually steps of 30 Watts were taken after every three minutes, but each time a load equal to 90, 180 or 270 Watts was reached, the time interval was extended to five minutes

No rules could be set for the maximum work level The latter was recorded together with data on heart rate systolic pressure, ventilatory minute volume and oxygen uptake The highest oxygen uptake observed was considered as the aerobic capacity

Good agreement between Leiden and Nijmegen can be understood as the result of intensive co operation of the investigators during their training The lack of agreement with the other centres is considered as a consequence of insufficient routine or of too great a variety of methods of investigation in spite of all efforts to brief the future participants at the study

It is concluded that the results of studies of the aerobic capacity can be compared only, if there has been intensive training and detailed exchange of information on methods of investigation

F I M Bonke and L N Bouman, *The early premature beat in the atrium*

Department of Physiology University of Amsterdam

In the isolated right atrium of the rabbit premature beats were elicited very early in the atrial cycle by means of a bipolar stimulation electrode placed on the atrium

The transmembrane potential of cells in the S A node was recorded with conventional glass micro-electrodes By means of unipolar electrodes the activation of the right auricle was recorded at two different points The interval between the last spontaneous activation of the atrium and the atrial stimulus exceeded the refractory period of the atrium by 10–20 msec This early stimulus elicits a premature beat that is followed by a shorter interval than the normal one between two spontaneous discharges of the S A node (Eccles and Hoff 1934) We observed a dissociation between the activity of the atrium and the S A node for in the impaled

pacemaker cell only one action potential was registered against two atrial complexes

Furthermore it was found that the conduction velocity inside the S A node at the beginning of the diastole is very slow This fall in conduction velocity of the early premature activation enables a re entry of the impulse into the atrium, causing an echo activation

Two types of early atrial premature beats were observed

type 1 the post extrasystolic pause is short and the pacemaker shifts within the S-A node (BONKE and BOUMAN, 1968)

type 2 the post extrasystolic pause is longer than in type 1 and no important shift of the pacemaker occurs

Type 2 occurs when the stimulus is given a few msec earlier in the atrial cycle than in the case of type 1

In our opinion the explanation of the difference between these two types lies in the fact that in the case of type 2 the impulse cannot enter the S-A node from the atrial side because these fibres are still refractory and the only possible entrance to the node is from the caval side

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BONKE F I M and L N BOUMAN Acta Physiol Neerl 15, 34 (1969)
ECCLES J C and H E HOFF, Proc Roy Soc (London) 115, 352 (1934)

I L Bonta and W R Buckett, *Pharmacological comparison between pancuronium bromide* [1] *and d tubocurarine*

Department of Pharmacology, Organon, Oss and Newhouse

From screening a series of bisquaternary steroids on the sciatic-gastrocnemius preparation of cats, pancuronium bromide was selected for an extensive pharmacological investigation Pancuronium bromide displays in the cat 10 times and in the dog 5 times the potency of d tubocurarine, whereas no difference in duration of action was found when the two compounds were tested at equally effective dose levels The anticholinesterase drug neostigmine readily and completely reversed the neuromuscular block produced by pancuronium bromide on the tibialis anterior muscle

[1] Pancuronium bromide is the approved name of 2β 16β dipiperidino 5α androstane 3α, 17β diol diacetate dimethobromide formerly known as NA 97

of the cat This and other experimental evidence enabled us to classify pancuronium bromide as a competitive type of neuromuscular blocker Nevertheless a number of differences as compared with d tubocurarine were found Firstly, pancuronium bromide is devoid of histamine releasing properties, with the consequence that at effective neuromuscular blocking doses it neither causes bronchoconstriction, nor a fall in blood pressure Secondly, a study on two kinds of skeletal muscles of the cat has shown that – unlike d tubocurarine – the blocking effect of pancuronium bromide was comparatively more pronounced on the tibialis than it was on the soleus muscle In contrast to d tubocurarine, pancuronium bromide is many times more effective in the cat than in the rat

Interaction studies with pancuronium bromide and anaesthetics have shown that the neuromuscular blocking effect of pancuronium bromide was reinforced by ether, thiopental or halothane It is known that the low blood pressure during anaesthesia is usually further depressed when d tubocurarine is administered This, however is not the case with pancuronium bromide In fact the blood pressure drop produced by halothane was to a certain extent counteracted rather than reinforced by pancuronium bromide A further analysis of this fact revealed that pancuronium bromide has a vagolytic effect which is apparently strictly limited to the cardiac effects of the vagus

A P C Bot and J P Schade, *Seizures during development of the chick brain*

Netherlands Central Institute for Brain Research, Amsterdam

Seizure activity in the developing chick brain has been studied upon systemic and local application of a number of agents such as methionine sulfoximine ouabain, tetrodoxin and cardiazol

Before during and after the seizure activity, a number of electrical parameters of the optic lobe have been determined (E E G, photic evoked potentials, direct cortical response) Characteristic differences between the various convulsive agents have been found as far as the time of onset of the convulsion the duration of the convulsion and the properties of the individual components of the convulsive activity were concerned

The results will be published in extenso in the near future

A J M Brakkee and A J H Vendrik, *Application of an indicator dilution technique in the study of a peripheral vascular bed in vivo*

Department of Medical Physics, University of Nijmegen

In order to obtain quantitative information about arterio venous shunt systems in a peripheral vascular bed, a double isotope dilution technique is developed which is applied in animal experiments For this purpose a mixture of ^{24}NaCl and ^{131}I albumin is injected into a supplying artery of the foot region of the hind leg of the dog Annulation of the saphenous vein enables the blood to pass a scintillation detector, a roller pump and a drop counter and to return into the same vein in the direction of the heart

The analysis of the dilution curves is based on the differences between the shunt and the capillary vessels with respect to exchange of small molecules

It appeared that with a silicon coating of the glass surface in the external loop, considerable errors are introduced as a result of the adsorption of albumin by this coating

The experiments indicate that also some other materials may introduce errors due to adsorption

J Bruinvels [1]) and T L Sourkes, *Monoamines and the harmaline induced hypothermia* [2])

Laboratory of Chemical Neurobiology Department of Psychiatry McGill University Montreal Canada

Harmaline administered to rats results in a decrease in body temperature (MARKOVIĆ and GIAJA, 1951) Pretreatment with α-methyldopa or α methyltyrosine 24 h before or parachlorophenylalanine 72 h before the administration of harmaline does not prevent the decrease in body temperature Pretreatment with reserpine

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1) On leave of absence from the Department of Pharmacology of the University of Utrecht The Netherlands recipient of a NATO Science Fellowship of the Organization for the Advancement of Pure Research (Z W O) Present address Department of Pharmacology University of Utrecht Vondellaan 6 Utrecht The Netherlands

2) This research was supported by the Medical Research Council of Canada

does not affect the temperature fall caused by harmaline However, administration of Ro-4 4602, a decarboxylase inhibitor, 24 h after reserpine treatment and 1 h before harmaline injection, prevents the fall in body temperature ($P<0\ 05$) Diethyldithiocarbamate like compound Ro 4-4602 prevents the decrease in body temperature ($P<0\ 05$) From these experiments it was concluded that noradrenaline is involved in harmaline induced hypothermia

Administration of harman and harmalol two congeners of harmaline also results in a decrease in body temperature This is in agreement with the results obtained by SCHMIDT and FAHSE (1964), who showed that MAO inhibition is not related to hypothermia in rats

Another explanation for the mechanism by which harmaline decreases body temperature is an increased binding of serotonin (LESSIN *et al*, 1967) and/or an inhibition of the uptake of noradrenaline (IVERSEN 1965) However, the percentages of free and bound serotonin and noradrenaline in rat brain, do not change after harmaline administration Ganglion blockade (hexamethonium mecamylamine) and adrenergic β blockade (propranolol) do not influence harmaline induced hypothermia Pretreatment with dibenzyline prevents the fall in body temperature caused by harmaline However, phentolamine was without effect

Harmaline harman and harmalol were found to antagonize the action of serotonin on the rat uterus *in vitro* If this effect should also occur in the C N S harmaline induced hypothermia may be the result of anti serotonergic properties of harmaline, which provokes an unbalance of monoamines in favour of noradrenaline

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SCHMIDT J and C FAHSE Acta Biol Med Germ 13, 607 (1964)

C H M Brunia *The influence of a task on the amplitude of the Achilles tendon reflex and the Hoffmann reflex*

Dr Hans Bergerkliniek, Breda

If the Achilles tendon reflex and the H reflex are simultaneously

evoked at both legs of one subject, the reflex-circuit in both cases is nearly the same only the muscle spindle plays no important role in the electrically induced reflex

The sensibility of this receptor is controlled by the fusimotor system and this again by supra-spinal mechanisms one of which is the reticular arousal system

When during a vigilance task both reflexes are evoked the amplitude of the mechanically induced one becomes significantly higher than at rest, whereas the H-reflex shows no pertinent change During a 20 minutes' task there is a slow diminution of the tendon reflex amplitudes, although they remain higher than at rest

Inhibitory mechanisms as a result of a habituation process seem to play an important role

G A Charbon, *The hepatic vasodilatation used as parameter for a bio-assay of parathyroid hormone*

Departments of Pharmacology and of Cardiovascular Surgery, Faculty of Medicine, University of Utrecht

During the preceding meeting (Acta Physiol Pharmacol Neerl, in press) a report was presented about experiments showing that parathyroid hormone (PTH) selectively induced a vasodilatation in the kidney and in the arterial bed of the liver, when injected into anaesthetized dogs A relatively crude parathyroid extract (PTE) containing about 10 % PTH, appeared to have an intrinsic activity similar to PTH

The results of a study investigating whether the augmentation of hepatic blood flow might be used as parameter for a bio assay of parathyroid hormone are given in the present paper The study was performed on anaesthetized dogs Blood flow was measured with a square wave electromagnetic flowmeter The technique used has been described previously (Arch Intern Pharmacodyn Thér 171, 1–11 (1968))

The dose response curve of PTE on hepatic flow was suitable to be transformed with the method of ARIËNS *et al* (Molecular Pharmacology, Ed E J Ariëns, Academic Press, London, 1964, pp 119–286) These transformations resulted in an acceptable linear relation between logit units and logdose When 3 dose response curves were made with one preparation on the same dog,

the third curve was shifted to the right when compared with the first and second curve For a bio assay such a shift is not attractive The first and second curve, however, were quite similar

The data justify the conclusion that a bio-assay of parathyroid hormone is feasible when using the augmented hepatic arterial flow as parameter Two dose response curves may be made on the same dog, making a cross-over design possible

P J Christen, P K Schot and E M Cohen, *Interaction of some cholinesterase inhibitors with aliesterase from rat plasma*

Medical Biological Laboratory RVO TNO, Rijswijk Z H, The Netherlands

Sarin (isopropyl methylphosphonofluoridate) is a potent inhibitor of cholinesterase and other esterases Using a microgel electrophoresis technique in combination with autoradiography, it was found (CHRISTEN and COHEN, 1969) that after the incubation during half an hour of 0 01 mM ^{32}P sarin with rat plasma all detectable radioactivity coincided with the aliesterase fraction In human plasma the radioactivity coincided with the albumin The amount of ^{32}P, bound to cholinesterase, was apparently too small to be detectable under our experimental conditions It could be shown that in rat plasma after incubation with 0 01 mM ^{32}P-sarin 35 % of the added ^{32}P was bound to protein In these experiments Sephadex gel filtration was used About the same figures were found after incubation with 0 01 mM ^{32}P soman (pinacolyl methylphosphonofluoridate) or $DF^{32}P$ (diisopropylphosphorofluoridate) Of the compound ^{32}P O ethyl S diethylaminoethyl methylphosphonothiolate 24 % of the ^{32}P [illegible]

to albumin during incubation with human plasma was also smaller than that of the other inhibitors used The amount of ^{32}P, bound to aliesterase in rat plasma, incubated with 0 01 mM ^{32}P-soman could be determined after polyacrylamidegel electrophoresis The place of the enzyme in the gels was indicated by staining with α naphtyl acetate and p-rosaniline The ^{32}P, coinciding with the aliesterase fraction, was estimated using liquid scintillation counting

From these experiments a concentration of catalytic centres in rat plasma of 2 8 μM was calculated

The ^{32}P bound to aliesterase after incubation of rat plasma with 0 01 mM ^{32}P sarin could easily be broken by lowering the pH of the incubation mixture (pH 7 6–7 8) to 5 5 This resulted in a rapid reactivation of the inhibited enzyme, as was found in experiments using a pH stat method with tributyrine as a substrate By paper chromatography it was found that ^{32}P isopropyl hydrogen methylphosphonate was released from the enzyme by this reactivation

REFERENCE

CHRISTEN, P J and E M COHEN Acta Physiol Pharmacol Neerl 15 34 (1969)

H Collewijn, *Dynamics of optokinetic movements in rabbit*

Dept of Physiology, Medical Faculty, Rotterdam

Optokinetic nystagmus is generally considered as a compensating mechanism, serving the purpose of optimal stabilization of the retinal image For an evaluation of the dynamics of this servo system in the rabbit the relation between angular velocity of visual surroundings (input) and of the eye (output) was investigated Encephale isolé rabbits were used, fully recovered from fluothane anaesthesia Ocular movement was measured with a drift free inductive scleral coil system This in combination with the rareness of spontaneous eye movements in rabbits permitted registration also of very low velocities By stimulating an immobilized eye and recording movement of the other, closed eye open loop" characteristics of the system were measured The input consisted either of constant rotation or sinusoidal movement of a striped drum, surrounding the animal

Gain (eye velocity/drum velocity) was between 0 7 and 0 9 for drum velocities from 0 03 up to 1°/sec At higher velocities gain decreased and maximal eye velocities were reached with appreciable delays The acceleration of the slow phase system was probably not better than 1°/sec² The same limit was manifest in sinusoidal movement *Gain decreased when drum accelerations exceeded* values around 1°/sec² This was not accompanied by substantial phase lag, though delays between 100 and 200 msec were commonly

seen Under open loop conditions, gain was markedly increased For drum velocities up to 0 1°/sec it was better than 10 and could even exceed 100 For drum speeds above 1°/sec gain decreased progressively to values below unity These results are roughly in agreement with closed loop data In monocular vision, backward drum rotation is inferior to forward rotation as stimulus The results indicate that the optokinetic system, without vestibular input, is a velocity servo with severely limited acceleration No evidence for genuine position correction was found

J H Dewaide and P Th Henderson, *Hepatic N-demethylation of aminopyrine in rat and trout*

Department of Pharmacology University of Nijmegen

Up to a short time ago it was suggested that aquatic vertebrates were not provided with a drug metabolizing capacity, because of the lipoidal character of gills and/or skin through which excretion of lipid soluble compounds could take place (BRODIE and MAICKEL, 1962) Recently, however, evidence began to rise that also in the livers of some aquatic animals there is a capacity, although very slight for drug metabolism (ADAMSON, 1967) Considering the *in vitro* assays for many drug metabolizing enzyme activities, the lack in uniformity in the methods reported is striking (SMITH *et al*, 1963, ORRENIUS 1965 ANDERS and MANNERING, 1966) For this reason conclusions about quantitative aspects of the enzyme activities concerned can hardly be drawn In this study an effort has been made to determine some optimal conditions for the *in vitro* assay of the hepatic N demethylation of aminopyrine in rat (Wistar) and rainbow trout (Salmo irideus) in order to obtain comparable enzyme activities The activities were determined in the 9000 g supernatants The procedures of tissue preparation and enzyme assay are described in detail elsewhere (DEWAIDE and HENDERSON, 1968)

In accordance with previous results (CREAVEN *et al*, 1967, DUTTON and MONTGOMERY 1958) in this study, too, a difference in temperature optimum, 37° for the rat and 25° for the trout, is evident Moreover it appears that in this respect the stability of the enzyme at different temperatures plays an important role The N-demethylase activity *in vitro* is constant only during a short period (10 min), especially if measured at 37° The *p*H-optima are

equal for the rat and trout and are approximately 8 For rat and trout N-demethylase the K_m-values for the substrate aminopyrine are $6\ 1 \times 10^{-4}$ M and $2\ 1 \times 10^{-3}$ M respectively and for the co substrate NADPH $4\ 0 \times 10^{-6}$ M and $2\ 2 \times 10^{-5}$ M respectively For measurements of maximal N-demethylase activities both of rat and trout as saturating concentrations of aminopyrine and NADPH have been used $16\ 7 \times 10^{-3}$ M and $8\ 8 \times 10^{-5}$ M respectively Adverse actions of *nicotinamide (5 to 50×10^{-3} M) and semicarbazide-* ($> 5 \times 10^{-3}$ M), substances commonly used in the test, have been observed The assay of the N-demethylase activities under the conditions outlined gives as comparable values a production of $15{,}0 \pm 2{,}6$ μMoles formaldehyde per hour per g rat liver and $1\ 1 \pm 0{,}3$ μMoles formaldehyde per hour per g trout liver

Drawing conclusions from these in vitro activities about the rate of N-demethylation of the drug *in vivo* – which is hazardous –, it can be stated that since in the trout studied the liver weight is *only about 1 % of the total body weight and in the rat about 4 %*, the N-demethylation capacity in the liver of the rat per unit of body weight is approximately 60 times higher than for the trout The question remains whether – besides the possibility that other mechanisms for disposition are present – the apparently low drug metabolizing capacity in fish will nevertheless be sufficient to dispose of compounds foreign to the body Relating the different *levels of drug metabolizing capacity in two totally different species*, like the homoiothermous rat and the poikilothermous fish to a basic parameter such as resting metabolism – which in its turn is related to the food intake and therewith to the degree of exposure to body-foreign compounds – might greatly reduce the difference in detoxificating capacity reported

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A J van Eick, *The use of the "Passive Avoidance Reaction"-test for the study of amnesic effects in rats*

Medical Biological Laboratory RVO TNO, Rijswijk Z H

In order to investigate the amnesic effect of drugs, the exploratory activity of rats was observed for 2 min in an apparatus consisting of an open illuminated compartment which has a connection with a smaller, closed, dark box Normally the rats spend most of the time in the small compartment When, however, the rats have received a painful electric shock in the small box at the end of the 2 minutes' period and are placed in the apparatus later on (test session), they avoid entering this compartment This is called the "Passive Avoidance Reaction" (PAR), (Kurtz and Pearl, 1960) When after a treatment the rats do enter the small box during the test session this is generally thought to be due to a disturbance of the memory process

Pentetrazol was administered on the day after the pain stimulus in two injections 50 and 30 mg/kg i p with one hour interval This treatment causes only slight convulsions During test sessions performed 1, 2 and 24 h after the last injection the PAR was found to be inhibited completely

To study the duration of the drug effect groups of rats were submitted to a test session, each subsequent day a different group, until 12 days after the drug treatment In the groups tested from day 1 till day 7 significantly more rats enter the small box than in control groups This shows that the amnesic effect of pentetrazol lasts for at least 7 days It was also found that if a pentetrazol treated animal is repeatedly confronted with the test situation, some form of reminiscence of the "pre-drug" pain stimulus returns in course of time

Significant inhibition of the PAR was also found with phencyclidin HCl 4 mg/kg i p (tested 2 h after injection) and 10 mg/kg i p (tested 4 h after injection) The drug was administered on the day after the pain stimulus

LSD 25 (0 001–0 06 mg/kg), scopolamine HCl (1–20 mg/kg), atropine sulphate (6 mg/kg) and amphetamine (2 mg/kg) were found to be ineffective

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T Eskes, J Braaksma and J Janssens, *Pressure recording in the non-pregnant human uterus*

Dept of Obstetrics and Gynaecology, St Lucas Hospital and Free University, Amsterdam

Pressure-recording with intra-uterine balloons is unreliable The wall of the balloon is an unknown factor in transmitting pressures The volume of the balloon can be a trigger for uterine activity

For this reason a small vinyl catheter (Becton, Dickinson Co Rutherford, New Yersey, U S A No VX 020), filled with a heparine-saline solution and provided with a small sutured sponge at the tip, is introduced into the cavity without any discomfort to the patient The catheter is connected to an Elema EMT 490 A pressure transducer and a Siemens one-channel Compensograph inkwriter (paper-speed 3 cm/min, 12 cm full scale) The intra-uterine catheter tip transmits the pulse-pressure waves of the complete uterus superimposed upon intra-uterine pressure waves (HENDRICKS, 1964, BENGTSSON, 1967)

The intensity and frequency and the complete pattern depends on the day of the cycle, the hormonal status and the presence or absence of gynaecological pathology A method for further quantitative statistical evaluation seems to be the total pressure area per unit of time, with a further subdivision of lowest and highest pressure values and the frequency of uterine contractions (ESKES, 1968)

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A L. Frankhuyzen and W de Jong, *Role of the adrenal gland in renin hypertension in the rat*

Rudolf Magnus Institute for Pharmacology, Medical Faculty, University of Utrecht, Vondellaan 6, Utrecht

Previous experiments in this institute (DE JONG, 1968) demonstrated that clip-induced renal hypertension is not dependent upon

the adrenal gland in rats maintained on 0 9 % saline MASSON *et al* (1966) reported that hypertension induced by subcutaneous administration of renin, a presumed pharmacological model for renal hypertension, did not occur in adrenalectomized rats maintained on 0 9 % saline The present report concerns the effect of renin administration on blood pressure of adrenalectomized male rats and also the influence of aldosterone and corticosterone on the effect of renin treatment

Partially purified renin was prepared from rat kidneys Conventional purification procedures, including acid denaturation and ammonium sulphate precipitation, were employed Ultrasonic desintegration of the kidney homogenate and preparative ultracentrifugation were used to increase the yield

The subcutaneous administration (twice daily) of this renin dissolved in 7 % gelatin (equivalent to ± 40 Goldblatt Units/day), induced hypertension in intact rats No change in blood pressure was observed in renin treated adrenalectomized rats maintained on 0 9 % saline Subcutaneous administration (twice daily) of aldosterone (6 μg/day) or corticosterone (60 μg/day) did not affect the blood pressure of adrenalectomized rats This treatment restored the renin induced hypertension in adrenalectomized rats

These results seem to indicate that a functional adrenal cortex is necessary for the development of renin hypertension in rats However, in later experiments it was found that renin hypertension also occurred in adrenalectomized rats when higher doses of renin were used

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MASSON G M C, K. AOKI, M MATSUNAGA and E R FISHER, Laboratory Investigation 15, 449–454 (1966)

M 't Hart, H Dienske, H A Udo de Haes and E. L Noach *Ethological investigation of the effect of some psychopharmacological drugs on the behaviour of rats*

Department of Pharmacology University of Leiden

The investigation was carried out on male rats of about 150–200 g Since the period of their greatest activity is during the evening, the rats were habituated to a different day night rhythm for two

T Eskes, J Braaksma and J Janssens, *Pressure-recording in the non-pregnant human uterus*

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A L Frankhuyzen and W de Jong, *Role of the adrenal gland in renin hypertension in the rat*

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Previous experiments in this institute (DE JONG, 1968) demonstrated that clip induced renal hypertension is not dependent upon

gram liver protein, was compared with the mitotic activity in the regenerating liver at the corresponding times After a short latency time following the subtotal hepatectomy, a decrease in the N-demethylase activity has been observed Minimal activity has been found about 50 h post-operatively. This minimum corresponds to the peak in the mitotic rate Sham-operation did not significantly affect the rate of N-demethylation, nor the mitotic activity in the livers of the control animals

The observation that the fall in N-demethylase activity and the increase in mitotic activity take place simultaneously, might support the view that a differentiated cell function like hepatic drug metabolism and active cellular proliferation mutually exclude each other

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M W van Hof, *Monocular vision in the rabbit*

Dept of Physiology, Medical Faculty, Rotterdam

Rabbits with one eye occluded were trained to discriminate striated patterns in a training box similar to the one described previously (VAN HOF, Vis Res 1966 p 89, VAN HOF en WIERSMA, Vis Res 1967 p 265) 8 animals learned to discriminate vertical and horizontal striations with one eye 45° and 135° striations with the other Each eye received 100 exposures per day After scores of 90 per cent or better were reached, interocular transfer was tested The "vertical horizontal" eye was exposed 50 times to the vertical horizontal striations followed by 50 exposures to the oblique striations During the same four days the "oblique striations" eye was exposed 50 times to the 45°–135° patterns followed by 50 exposures to the vertical horizontal striations Interocular transfer was found to be very poorly developed Only in two out of eight animals transfer of vertical versus horizontal discrimination was found One other animal showed some transfer

weeks, after which the experiments were carried out during the afternoon, when activity was at its highest The behaviour of the rats was observed and analysed, the patterns of behaviour were defined The behaviour was observed "in social situation" (in the cage is also another rat) and "in non social situation" (only the test animal is in the cage)

The following drugs were administered 2 or 5 mg/kg amphetamine (stimulant), 25 or 50 mg/kg isocarboxazid and 10 or 20 mg/kg amitriptyline (antidepressant drugs) Rats injected with amphetamine were less aggressive, more explorative and less eager to eat Rats injected with isocarboxazid were less aggressive, less explorative and more eager to eat Compared with one single treatment, chronic treatment with isocarboxazid did not yield any new results Amitriptyline was effective only in non social situation (the number of all patterns was diminished) The drug effects were mostly dose dependent It was possible to distinguish between different doses of one single drug and between the different drugs isocarboxazid, amphetamine and amitriptyline in "blind" experiments

P Th Henderson and J H Dewaide, *N-demethylation of aminopyrine in regenerating rat liver*

Department of Pharmacology, University of Nijmegen

In the course of liver regeneration after partial or subtotal hepatectomy structure and chemical composition of rat liver microsomes become modified in a characteristic way (von der Decken and Hultin, 1958, Bernhard and Rouiller, 1956) Fouts *et al* (1961) reported a decrease in microsomal drug metabolism in regenerating liver after partial hepatectomy It is striking that also liver tumors, foetal livers and livers of newborn mammals – growing tissues which show a relatively high mitotic activity – have only a poor capacity of metabolizing body-foreign compounds

In the present study a correlation between drug metabolism and mitotic activity has been investigated in regenerating rat liver In adult male rats the oxidative N demethylation of aminopyrine has been determined *in vitro* at different times after subtotal hepatectomy (70–75 %) The assay procedure has been described previously (Dewaide and Henderson, 1968) The N demethylase activity, expressed as the amount of formaldehyde produced per

M J Janse, R Th van Dam and A B. M van der Steen, *Adaptation rate of the ventricular refractory period of the heart to sudden alterations of heart rate*

University Department of Cardiology and Clinical Physiology, Wilhelmina-Gasthuis, Amsterdam

The duration of the refractory period depends on the heart-rate. It is short at a fast rate and long when the heart beats slowly.

We investigated the speed of adaptation of the refractory period upon sudden alteration of the heart rate in canine hearts *in situ*, in which a total A V block was produced by electrocoagulation The heart was driven regularly, and for the steady state at two rates, 600 and 300 msec basic intervals, strength-interval curves were determined

In the area of the cardiac cycle between the ascending parts of both curves, several test-intervals were selected

After sudden doubling of the heart rate, from 600–300 msec basic interval, test stimuli of 1 5 times diastolic threshold intensity were delivered at one of these test-intervals, following every, sometimes every second, basic stimulus The number of beats of the new rate occurring before the heart responded to the test stimulus was counted This procedure was repeated for the other selected test-intervals

Following sudden doubling of the heart-rate ("on-effect") the refractory period shortens according to a more or less exponential curve In the first approximately 15 beats, 50 % of the total shortening occurs, the final value is reached after more than 500 beats The initial segment of the curve displays a characteristic damped oscillation between longer and shorter refractory periods in subsequent beats This effect was also observed in the propagation of the premature beats elicited by the test-stimuli, studied with multipolar intramural needle-electrodes

With a similar technique, by suddenly changing from the high to the low heart rate ("off-effect") we obtained approximately the same, but inverted curve, in which, however, the initial oscillation was less clear than for the "on-effect" Control tests proved that the repeated application of subliminal test-stimuli during the refractory period did not affect the observed values

Our findings suggest that the rate of adaptation is dependent

of the oblique striations discrimination At the present time experiments are in progress which indicate that the rabbit is able to learn a discrimination task with one eye which is conflicting with a task learned with the other eye

F C Jager, K D Njio, J A Verbeek and R O Vles,
Dose response effects of Vitamin E in ducklings

Unilever Research Laboratory, Vlaardingen

Ducklings react readily to lack of vitamin E and are, therefore suitable test subjects for investigating the requirement of this vitamin

In order to calculate accurately the extent of this requirement for a number of different criteria, dose responses were determined

To this end one day old Peking ducklings were kept on a 35 cal % lard diet for 4 weeks Per group ($n = 10$) doses of vitamin E varying from 0 5 to 256 mg D α tocopherylacetate per kg food were administered The effects of these doses were determined for the following criteria weight, mortality, haemolysis (*in vitro*) myopathy of skeletal muscles and gizzard serum creatine phospho kinase, serum glutamate oxalacetate transaminase, serum lactic dehydrogenase, composition of serum proteins and differential count of white blood cells

Suitable criteria for determining vitamin E requirement, appeared to be the histologically determined degree of myopathy of the skeletal muscles and the activity of the lactic dehydrogenase in the serum Using these criteria the requirements were found to be higher than with any of the other parameters Furthermore, both criteria showed an inverse linear log dose response relation To prevent myopathy 20 mg D α tocopheryl acetate per kg food was required

The effect of vitamin E on the composition of the blood proteins was striking The globulins (α_1, α_2 and γ) showed a linear decrease and the albumin a linear increase, in relation to the log dose vitamin E

After 4 weeks, the weight increase showed an optimal effect with 64 mg D α tocopheryl acetate per kg food

In contrast to lard no myopathy occurred with a corresponding maize oil diet Apparently maize oil contains by nature sufficient tocopherol to cover the requirement of vitamin E

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H P Kimmich and F Kreuzer, *Catheter P_{O_2} electrode with low flow dependency and fast response*

Department of Physiology, University of Nijmegen

The two central requirements of polarographic P_{O_2} electrodes to continuously record transient changes of P_{O_2} in moving fluid flow independency and fast response, oppose each other (Schuler and Kreuzer, 1967) A catheter oxygen electrode (diameter 2 mm) is described here which offers a good compromise between these two properties and makes the continuous recording of arterial P_{O_2} fluctuations with respiration and heart beat possible The cathode is a platinum foil 2 5 to 3 μ thick sealed between a tube and a cylinder (polyvinyl chloride or glass) forming a ring at the tip of the electrode The cylinder shaped silver anode also carries the membrane held by a stainless steel or silver ring The electrolyte is a phosphate buffer solution adjusted to *p*H 8 The current-voltage curves (polarogram) show a broad horizontal plateau over a wide range of voltage at all levels of O_2 up to 100 % O_2 The calibration line is linear and passes through the origin in most cases when the electrodes are polarized with −0 9 volts The current output of 0 5 to 0 7 μA for 100 % O_2 at 37° C with a 6 μ Teflon membrane is equivalent to that of a single wire cathode of 100 μ diameter The stability is 0 5 % over a period of 10 h and 2 % during several days The electrode is insensitive to pressure up to 0 3 kg/cm² and to acceleration below 50 m/sec² The flow dependency in blood at 37 °C is such that the reading remains within 2 % of the full value at high flow for linear velocities above 5 cm/sec with a 6 μ Teflon membrane and above 1 25 cm/sec for a 12 μ Teflon membrane The response time for 95 % deflection is 0 3 sec in gas and 0 4 sec in blood with a 6 μ Teflon membrane, it increases in blood to 1 sec by decreasing the velocity to 10 cm/sec The sensitivity of the output to changes of temperature is comparable to that of other polarographic electrodes

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on the number of beats rather than on the time during which the new rate exists

W de Jong, *Experimental renal hypertension in intact and in adrenalectomized rats*

Rudolf Magnus Institute for Pharmacology, Medical Faculty, University of Utrecht, Vondellaan 6, Utrecht

Development and maintenance of renal hypertension was studied in male white rats Hypertension was induced via a clip (0 30 mm internal diameter) on the left renal artery Blood-pressure was determined by a tail sphygmographic method (DE JONG, 1968) The rise in blood pressure started 2–5 days following application of the clip and gradually increased, reaching a maximum (± 200 mm Hg) 2–3 weeks later Neither severe reduction in kidney weight nor macroscopic infarction of the kidney was necessary for the development of this hypertension

Removal of the clip or extirpation of the left kidney of the hypertensive rats caused a fall in blood pressure to the control level This occurred in the acute stage of the renal hypertension (12 days) as well as in the chronic stage (45 days) A sham operation did not affect the blood-pressure of hypertensive rats Removal of the clip or extirpation of the left kidney of normotensive rats did not affect the blood pressure either

Renal hypertension following application of a clip on the left renal artery of adrenalectomized rats developed in a manner similar to that observed in intact rats Established states of renal hypertension of 12 days' and 45 days' duration were not materially affected by adrenalectomy The adrenalectomized rats were maintained on 0 9 % saline

It is concluded that these results demonstrate the renal nature of this type of hypertension in rats in the acute and in the chronic stages and also show that this renal hypertension is not dependent upon the adrenal gland

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J A J Klijn and G P M Horsten, *Centrifugal influences on the electroretinogram*

Department of Neurophysiology, University of Nijmegen

In the relation between the amplitude of the E R G (a+b potential) and that of the dark adaptation after standard blinding it was possible to induce significant changes by bipolar stimulation of the Lateral Geniculatum Body (200 C/sec, 3 M A)

An increase in the amplitude was caused by the stimulation in all stages of the dark adaptation The C F F values also showed a rise when the Lateral Geniculatum Body was stimulated

In all cases the increase in amplitude and C F F value was highly significant as a consequence of stimulation ($p < 0\ 01$)

A van Langevelde and E L Noach, *Metabolism of RNA in rat brain cortex during incubation*

Dept of Pharmacology University of Leiden

Slices of rat brain cortex were able, while incubated, to convert orotic acid into uridylic acid and incorporate the latter into RNA Incubation was carried out in manometric vessels containing Krebs Ringer solution and pure oxygen as gas phase according to Mc-

[illegible]

bation fluid, could be examined

Slices of 0 3 mm or 0 5 mm thickness were used First, outer or pial and second, inner slices could be cut from cerebral cortex if both were 0 3 mm The rate of oxygen uptake was higher in

E van der Kleijn, *Macroautoradiography of ataractics of the meprobamate-group*

Department of Pharmacology, University of Nijmegen

Certain homologues of meprobamate have a number of pharmacological actions in common with meprobamate and are also used in the treatment of anxiety and psychic tensions However, the N-isopropyl- and N-*n*-butyl derivatives (carisoprodol and tybamate respectively) are more lipophilic than the parent compound, as shown by partition coefficients and other physico chemical parameters (DOUGLAS *et al*, 1964) The effect of this increased lipophilia on the distribution of the drugs has been studied by the "whole body" autoradiographic technique of ULLBERG (1958), the compounds being labelled with ^{14}C in the unsubstituted carbamate group

Meprobamate is uniformly distributed in mice, but brain, thymus and body fat show a relatively low concentration, compared to that in the blood, myocardium, liver and muscles (EWALDSSON, 1963, VAN DER KLEIJN, 1967)

By qualitative comparison of the macroautoradiograms of mice intravenously injected with meprobamate, carisoprodol and tybamate, it appears that lipid solubility plays an important role in the rate of penetration of the drug into brain tissue Thus, uptake of meprobamate into the central nervous system is slow and slight, whereas carisoprodol and especially tybamate show high central concentrations within five minutes after intravenous administration However, the brain levels of carisoprodol and tybamate decrease rapidly and ten minutes after administration the whole body distribution patterns resemble that of meprobamate

No marked differences in the body distribution of the three compounds have been observed following their rectal administration and this may be due to their rapid metabolic conversion to hydroxylated and dealkylated compounds When rapid onset of action and minimum metabolic conversion are required, rectal administration offers no advantages over the oral route, particularly for compounds which have a short biological half-life and are active only when present in relatively high plasma concentrations In both cases the rate of absorption and release of the drug are limiting factors

These figures should be corrected in the following way

a) From data of MAAS (1967) we know that the error of pH_e when measured in whole blood using a saturated KCl bridge, is 0 01 pH-unit, and that the error of pH_i, when measured in hemolyzed erythrocytes, amounts to 0 03 pH units The corrections indicated above are to be added to the measured values A further correction arises from the residual plasma in the hemolysate in our procedure about 10 % of plasma are left behind between the erythrocytes From our experience (DE LEEUW *et al*, 1967) this causes an apparent increase of pH_i of 0 01 pH-unit Calculation of these factors gives $r_H = 0\ 69$

b) The concentrations of chloride should be calculated per litre of water in view of the high protein content of plasma and especially of cells For this we introduced fixed water contents of 93 and 71 % (v/v) respectively

The distribution coefficient of chloride is the ratio between the activities of Cl in cells and plasma $r_{Cl} = a_{Cl,i}/a_{Cl\ e}$ This ratio is not the same as the concentration quotient because of the different activity coefficients in cells and plasma

$$a_{Cl\ i}/a_{Cl\ e} = (f_{Cl\ i}/f_{Cl\ e})(c_{Cl,i}/c_{Cl\ e})$$

The Debije-Hückel formula in its limiting form ($-\log f = A\sqrt{c}$, for diluted solutions of univalent ions) enables us to approximate $f_i/f_e = 0\ 955$ (with $A = 0\ 5$, $C_i = 0\ 19$ and $c_e = 0\ 16$) Taking into account these considerations r_{Cl} becomes 0 63

Our final results are

$$r_H = 0\ 69 \pm 0\ 03 \text{ (mean} \pm 1\times \text{stand dev)}$$

$$r_{Cl} = 0\ 63 \pm 0\ 06 \text{ (mean} \pm 1\times \text{stand dev)}$$

In view of the number and the uncertainties of the described corrections, we think that this discrepancy between r_H and r_{Cl} does not prove the invalidity of the Donnan theory for the electrolyte distribution between plasma and blood cells

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DE LEEUW R J M, Miss M L. HAMELINK, K A FOEST and B F VISSER, 8e Fed Verg Med Biol Ver, Groningen 1967 Acta Physiol Pharmacol Neerl 15, 63 (1969)

0 3 mm slices than in 0 5 mm slices At equal rate of oxygen uptake pial slices turned out to incorporate more than twice the amount of ^{14}C RNA that was incorporated by inner slices

In order to gain an insight into the partition and specific activity of RNA in the various subcellular fractions, homogenization and centrifugation were carried out Labelled RNA was almost exclusively found in the nuclear fraction as was confirmed by auto radiographic observation of the slices Incubation for 2 h influences the distribution of the subcellular fractions and the RNA in them when compared to homogenized fresh cortex tissue, in such a way that most dry matter and RNA disappear from the microsomal fraction and are found in the nuclear fraction This distribution seems to be restored to normal fresh tissue conditions by adding 15 % rat serum to the incubation saline

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R J M de Leeuw, Miss M L Hamelink and B F Visser, *The distribution of H^+ and Cl^- between blood plasma and erythrocytes*

Longfunctielaboratorium, Academisch Ziekenhuis, Utrecht

The distribution of H^+ and Cl^- between plasma and cells was studied in anaerobically drawn venous blood of 10 healthy persons, 5 women and 5 men

The activity of the hydrogen ions in plasma ($a_{H\ e}$) and in cells ($a_{H\ i}$) was determined by pH-measurement in whole blood and in hemolyzed erythrocytes with the micro glasselectrode and the saturated KCl-calomelelectrode, the whole being thermostatted at 38 °C

For the determination of the distribution of chloride we measured the Cl concentration in deproteinized plasma ($C_{Cl\ e}$) and deproteinized whole blood ($C_{Cl\ b}$) by potentiometric titration, together with the hematocrit value (Hc)

The provisional results were

a) $a_{H\ e}/a_{H\ i} = 0\ 67 = \text{antilog}\ (pH_i - pH_e)$

b) $C_{Cl\ i}/C_{Cl\ e} = 0\ 50 = 1 - (1 - C_{Cl\ b}/C_{Cl\ e})/Hc$

Chromatography was done on thin layers of silicagel GF_{254} either by developing three times with chloroform methanol 95 5, or twice with chloroform methanol acetone 80 10 10 The former solvent system gave a better separation of the rapidity migrating metabolites, with the latter the slowly moving compounds were better dissolved

The spots were located by U V light and by spraying with a 1 % $Hg_2(NO_3)_2$ solution Keto groups were identified with 2,4-dinitro-phenylhydrazine

From rabbit urine 8 spots were obtained with this method Three corresponded with nor-, 3 hydroxy-nor- and 3 keto-nor hexobarbital, obtained from the urine of rabbits injected with nor hexobarbital The other five contained metabolites with an intact 1-methyl barbiturate ring, judging from their U V spectrum, 2 of these could be identified as 3 keto- and 3 hydroxy hexobarbital

Incubation of the rabbit urine, after continuous extraction with ethylacetate with β-glucuronidase produced 3 hydroxy- and 3-hydroxy nor hexobarbital indicating the presence of the corresponding glucuronides

From mouse urine the same metabolites could be extracted However in extracts of mouse urine the demethylated compounds were present in relatively much smaller amounts than the other metabolites, as compared with rabbits As yet no glucuronides could be demonstrated in mouse urine

R Peset, H Heemstra, C R H Wildevuur, F Gimeno and G J Tammeling, *Effect of unilateral positive pressure breathing on ventilation in dogs before and after lung reimplantation*

Departments of Chest Diseases Respiration Physiology and Thoracic Surgery University of Groningen

Unilateral positive pressure during bronchospirometry is achieved by placing a weight on one of the spirometer bells In one series of experiments a weight of 0 5 kg, producing a pressure of +6 cmH_2O in the breathing circuit was used Under these conditions two types of phenomena take place One type is mechanical and consists of an inspiratory shift of the breathing level of the lung connected to the circuit under positive pressure The breathing level of the contralateral lung is shifted to the expiratory side

C P J J M M Legein and M W van Hof, *The ERG of the dark reared guinea pig*

Dept of Physiology, Medical Faculty, Rotterdam

In previous studies (VAN HOF and USAMI, 1968 USAMI and VAN HOF, 1968) it was found that the *b* wave of the ERG of guinea pigs deprived of light during the first three months after birth is some 50 % smaller than in animals reared under normal circum stances This was confirmed in the present experiments Contrary to results obtained by ZETTERSTROM (1955) and BAXTER and RIESEN (1961) in the cat it was found that recovery by light exposure following the light deprivation is a comparatively slow process A 10 days' period of additional light exposure is not sufficient to normalize the *b* wave's amplitude

At the moment experiments are carried out to answer the question whether this effect of light deprivation is typical for the early post natal period Preliminary results indicate that a three months' light deprivation in the adult guinea pig also decreases the *b* wave s amplitude considerably

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ZETTERSTROM B The effect of light on the appearance and development of the ERG in newborn kittens Acta Physiol Scand 35 272 279 (1955)

J Noordhoek and B J Gerritsen *Chromatographic separation of hexobarbital metabolites in rabbit and mouse urine*

Department of Pharmacology Free University Amsterdam

After oral administration of 400 mg/kg hexobarbital Na to rabbits and 300 mg/kg to mice the urine was collected during 24 h After the addition of 20 % (w/v) NaCl and acidifying with HCl to $pH = 3$ the urine was shaken twice with two volumes of ethylacetate The extract was dried over Na_2SO_4 and concentrated at 40 °C under vacuum

The measurement of blood flow during slight exercise was rather simple, in spite of the movement artefact During heavy exercise we encountered some problems by insufficient venous occlusion, even if we used the correction described by Barcroft and Dornhorst We had the impression that blood flow during heavy exercise was lower than blood flow measured directly after work

Increase in frequency, counter weight and angle of movement resulted in an increase in blood flow Combination of these three factors caused the highest blood flow of 80 ml blood/ml tissue per minute The blood flow in rest was about 3 ml blood/ml tissue per minute The relation between work and blood flow was nearly rectilinear

As a tentative conclusion we can state that work on a foot ergometer is a good method to stimulate blood flow in the calf

REFERENCES

THULESIUS O A foot ergometer for graded muscular exercise Scand J Clin Lab Invest 15 550 (1960)

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H van Rees, E L Noach, A Udo de Haes de Bakker, A van Tilburg and S K Westenberg, *The resorption of diphenylhydantoin (Dilantin®) from the intestine*

Dept of Pharmacology University of Leiden

NOACH *et al* (1958) showed that after intravenous injection dilantin appeared in the intestine from where, however, it was reabsorbed almost completely

Attempting to localize these secretion and resorption processes, NOACH and VAN REES (1964) injected dilantin intravenously in rats in which the intestine was ligated at different sites From a comparison of the dilantin contents of these pieces of intestine with those from sham-operated animals it was concluded that there must be at least three passage cycles Furthermore the hypothesis was posed that secretion must be dominant in the duodenum and resorption in the remainder of the small intestine

This hypothesis was tested by determining *in vivo* the disappearance rate of dilantin which was injected intraluminally

The halfclearance time after administration of 6 mg dilantin

By removing the weight the breathing level of both lungs is shifted in opposite direction, but the level of the lung under positive pressure does not usually return to its previous level These phenomena are similar in both intact and reimplanted lungs The *second type of phenomena taking place is related to the integrity* of the pulmonary stretch reflexes By applying the positive pressure to the intact lung, respiratory frequency decreases at once and deep respiratory movements followed by long expiratory pauses *usually occur When the positive pressure is applied to the re*-implanted lung no change in frequency is observed, indicating that the stretch reflexes are abolished In other experiments progressively increasing weights were placed on one of the spirometer bells *Positive pressures ranged from +0 5 to +16 0 cmH₂O It was* observed that with relatively low pressures (+7 cmH_2O) the intact lung showed periods of apnoea of 1 min or longer The reimplanted lung on the contrary went on ventilating at the same frequency *with relatively high pressures (+16 cmH_2O) The present method* gives more information and is simpler than others previously mentioned in the literature (MARSHALL and GUNNING, 1966, PORTIN e a, 1960, and TRUMMER, 1964)

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MARSHALL, R and A J GUNNING, J Surg Res 6, 185 (1966)
PORTIN, B A, C L RASMUSSEN, J D STEWART and M N ANDERSEN, J Thoracic and Cardiovasc Surg 39, 380 (1960)
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J Pool, J J W Edgar and C V van Gool, *Blood flow through the human calf during dynamic exertion*

Dept of Cardiology, University Hospital, Leiden

As a natural method of stimulation of blood flow through the calf we used a foot ergometer as described by Thulesius We modified it by substituting the braking spring by an adjustable counter weight The work of the calf muscles could be regulated by changing the frequency, the counter weight and the angle of movement in the ankle joint

Blood flow was measured by venous occlusion plethysmography according to Whitney, during and directly after work The optimum pressure for venous occlusion was about 60 cm of water

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In similar circumstances, DMPP action was potentiated by guanethidine, and was blocked by bilateral adrenalectomy OG 30 was also found to increase the intraluminary pressure in urinary bladder and intestine of dogs and the isolated guinea pig ileum These effects were proved to result from parasympathetic ganglia-stimulation In higher amounts, OG 30 transiently blocked the neuromuscular and ganglionic transmission like DMPP, but unlike the reference compound OG 30 had no stimulatory effect on the isolated guinea pig heart The LD_{50} of OG 30 in mice by i v and i p routes was found to be 10 25 and 57 75 mg/kg, respectively. It was, however, non toxic by the oral route owing to a lack of absorption On the basis of toxicity pattern, and the ineffectiveness of hexamethonium in completely preventing OG 30 lethality, it is argued that the compound caused death not by its autonomic effects but mainly by blocking neuromuscular transmission In conclusion, OG 30 resembled DMPP in several respects although some differences have been outlined

J P Schadé and G Mulder, *Self-stimulation physiological and pharmacological aspects of septum stimulation in rats*

Netherlands Central Institute for Brain Research Amsterdam

128 rats were used for an investigation of the characteristic phenomena of septum and hypothalamus-stimulation in rats

Bipolar implanted electrodes were used unilaterally and bilaterally The first part of the investigation consisted of the determination of stimulus parameters and the determination of base levels The influence of a number of psycho active drugs was investigated (amphetamine chlorpromazine, amitryptiline etc) Concomitantly a number of behavioural parameters were analysed before, during and after the septal stimulation

The results will be published in extenso in the near future

P F Sonneville, *Indirect action of dopamine*

Rudolf Magnus Institute for Pharmacology Medical Faculty, University of Utrecht, Vondellaan 6 Utrecht

Besides α and β adrenergic properties dopamine still has other actions that so far have not been tested in isolated organ systems

was almost 3 h for the duodenum, 6½ h for the small intestine distal of the duodenum and about 11 h for the colon

Preliminary results of experiments *in vitro*, incubating pieces of duodenum in Tyrode solution with different dilantin concentrations inside and outside the intestine, indicated that the discrepancy between intravenously and intraluminally injected animals may be due to a high secretion rate of fluid into the duodenum

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P R Saxena, *Pharmacological studies with a new ganglion stimulating agent*

Department of Pharmacology, N V Organon, Oss

Out of a number of compounds tested for cardiovascular activity in anaesthetized cats, 2 imino 1,3-diaza-4-carboxyethyl cycloheptane hydrochloride (OG 30) was found to produce pressor effect along with a strong contraction of the nictitating membrane for short periods at 0 25 to 1 mg/kg, i v dose The mechanism of the aforesaid action, and other pharmacological effects of the compound OG 30 were studied in cats dogs, and in isolated preparations in *comparison with 1 1 dimethyl 4 phenylpiperazinium iodide*(DMPP) The pressor effect and the contraction of the nictitating membrane elicited by either compounds in cats were blocked or reduced by phentolamine, hexamethonium, N-N diisopropyl-N'-isoamyl-N'-diethylaminoethylurea hydrochloride (P 286) and reserpinization, but were not diminished by cocaine, hemicholinium spinal section or atropine Furthermore, upon direct intra arterial injection to the superior cervical ganglion *both OG 30 and DMPP caused nictitating* membrane contraction which was blocked by hexamethonium or destruction of the ganglion Thus, OG 30 appeared to resemble DMPP as nicotinic ganglionic stimulant However, as bilateral adrenalectomy did not appreciably affect the pressor response to OG 30, while subsequent bilateral sympathectomy, and guanethidine largely abolished it, it must be concluded that the compound caused *a release of catecholamines* by stimulating mainly the sympathetic ganglia and only to a small extent the adrenal medulla

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A B M van der Steen, R Th van Dam and M J Janse, *Electrophysiological aspects of paired stimulation*

University Department of Cardiology and Clinical Physiology, Wilhelmina-Gasthuis, Amsterdam

During paired stimulation, every pair of electrical stimuli to the heart results in two electrical activations and in one mechanical systole The positive inotropic effect has been studied more extensively than the electrophysiological parameters We studied the effect of paired stimulation on refractoriness and conduction in canine hearts The spread of both activations in Langendorff-perfused canine hearts was studied with ten-polar intramural needle-electrodes, arranged in vertical and horizontal planes in the entire heart The refractory period of the first beat of every pair shortens during paired stimulation, it lengthens again as the second stimulus occurs earlier Following an early second stimulus, the second activation is propagated in partially refractory myocardium During stimulation at the epicardial surface of the right or left ventricle conduction occurs mainly via myocardium, resulting in a relatively long duration of complete ventricular activation of the first beat of every pair, e g 110 msec, as compared to approximately 50 msec during sinus rhythm The second activation takes even longer than the first one, e g 140 msec, its QRS complex differs in width and shape Conduction of the second impulse is slower and follows a different course in ventricular areas distant from the stimulus site This is due to conduction in refractory myocardium and probably also to longer lasting refractoriness of the specialized conduction system

In a 6 week-old myocardial infarction, produced by ligation, both activations are even more retarded In the infarcted area, the surviving myocardium conducts the activation wave non-uniformly and slowly Local activation sometimes takes 20-30

It was found that the fundus preparation of the rat contracted under the influence of dopamine, whereas norepinephrine and isuprel caused relaxation in this system The 5 HT antagonist methysergide was able to block the action of dopamine

Repeated administration caused tachyphylaxis, which could be overcome by incubating with 5 HT

From these facts it is concluded that dopamine might act by releasing 5-HT in the tissues

D Spruit and K E Malten, *Increase of the water vapour loss of human skin following exposure to liquid water*

Department of Dermatology, R C University, Nijmegen

With a ballpoint a number of sites to be measured on the volar aspect of the fore arm have been marked Daily measurement of the water vapour loss from these sites is possible using an electrolytic water analyser and dry nitrogen gas being lead through a cup pressed upon the site of the skin to be measured (SPRUIT and MALTEN, 1965) The influence of various substances applied to the marked sites on the fore arm for one hour per day is measured the day after the exposure took place by determining the increase of the water vapour loss compared with the water vapour loss of uninvolved normal skin sites (SPRUIT and MALTEN, 1968) The exposures may be repeated on subsequent days mostly six successive days By the daily measurement of the water vapour loss from the exposed sites, plotted on semilogarithmic paper the way the skin adapts itself to the influence of the exposures and regenerates after its withdrawal has been followed (MALTEN *et al* in the press) In a series of recent experiments the influence of repeated exposure to water has been measured In some environmental circumstances (usually in summer) no effect at all could be detected in other environmental circumstances (*e g* in autumn) a moderate increase of the water vapour loss of normal skin and of slightly injured skin was measured It is suggested that the measured increased water vapour loss in summer (insensible perspiration) (HEERD and OPPERMANN, 1966) is caused by increased exposure of the skin to water and sweat

6 % dextran 75 000 (Macrodex) in 5 % glucose 10 minutes post-inj the volume determinations showed a small decrease in the first example, a large one in the second, and a small increase in the third Comparable results were obtained when the means of the haematocrit values of at least five rabbits were calculated After injection of 5 % glucose, not only glucose but also, to a certain degree, water is resorbed, after dextran, especially after rheomacrodex, a back resorption of tissue fluid due to the hyperoncotic action of the dextran, appears From the resistance it could be calculated that the conductance of this fluid corresponds with that of a physiological salt solution

In some experiments the glucose was replaced by salt solution (0 9 % NaCl) Comparable results, as in the glucose set, were obtained After pure salt solution no real volume-expansion appears in contrast to the dextran solutions

A gelatine product in a salt solution, on the other hand, causes no real plasma expansion These relations, observed after the T 1824 method, could be confirmed by haematocrit-determinations, in which the means of at least five rabbits were used In relation to the Hct as well as to the resistance the gelatine product behaves more like a pure salt solution than dextran solutions do

Z Turek and F Kreuzer *Comparison of two methods for estimating pulmonary capillary back pressure of CO during hypoxia*

Department of Physiology University of Nijmegen

In the determination of the pulmonary diffusing capacity for CO (D_{LCO}) with the steady state method, the back pressure of CO in pulmonary capillary blood (P_{cCO}) cannot be disregarded in many cases The present communication deals with the estimation of P_{cCO} during breathing a hypoxic mixture FORSTER *et al* (1957) calculated P_{cCO} with a modification of the method of SJOSTRAND (1948) while breathing pure oxygen, which might be questionable in an investigation concerning the influence of hypoxia For this reason we have applied to hypoxic conditions the breath holding method described by JONES *et al* (1958) for room air breathing In this method it is assumed that the partial pressure of CO in the alveolar air after breath holding should approximate the mean partial pressure of CO in pulmonary capillary blood

msec during sinus-rhythm or following the first stimulus; following the second stimulus this takes up to 40–50 msec.

In a fresh myocardial infarction, conduction is still slower and paired stimulation invariably initiates ventricular fibrillation, probably by re-entry.

This prohibits clinical application of paired stimulation in cardiogenic shock associated with acute myocardial infarction.

W. F. H. Ströer, *Changes in plasma conductance after administration of infusion fluids*

Pharmacological Dept. of Provite Products N.V., Amsterdam

The determination of the intravascular plasma volume with the aid of Evans' blue dye (T 1824) in rabbits after administration of 10 ml/kilo of infusion fluids, is not always reliable. It was thought that the conductance (*C*) of the plasma and its reciprocal value the resistance (*R*) might produce a valuable control. Therefore the latter was determined in mixtures of plasma with different percentages of these fluids, and curves, showing the relation between the latter and *R*, were constructed. The agreement between the observed *R* values and those calculated by means of the law of Ohm was remarkable.

According to this law:

$$\frac{1}{R} = \frac{1}{\frac{a_1+a_2}{a_1}R_1} + \frac{1}{\frac{a_1+a_2}{a_2}R_2}$$

a_1 and a_2 are the volumes of the fluids with resistances R_1 and R_2. These curves can also be used to determine the percentage glucose or dextran-glucose of a fluid, when its resistance is known, as was shown in three examples.

1) In a rabbit, 10 min after administration of a 5 % glucose solution, two thirds of the glucose are resorbed. At that moment the glucose percentage of the plasma, determined by a corrected Hagedorn-Jensen method, agrees exactly with that looked up in the afore-mentioned diagram with the aid of the *R*, calculated by applying Ohm's law to the observed resistances.

2 and 3) Comparable results were obtained in two other rabbits after injecting *10 % dextran 40 000* (Rheomacrodex) or

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Z Turek and F Kreuzer, *Comparison of two methods for estimating pulmonary capillary back pressure of CO during hypoxia*

Department of Physiology University of Nijmegen

In the determination of the pulmonary diffusing capacity for CO (D_{LCO}) with the steady state method, the back pressure of CO in pulmonary capillary blood (P_{cCO}) cannot be disregarded in many cases The present communication deals with the estimation of P_{cCO} during breathing a hypoxic mixture FORSTER *et al* (1957) calculated P_{cCO} with a modification of the method of SJOSTRAND (1948) while breathing pure oxygen, which might be questionable in an investigation concerning the influence of hypoxia For this reason we have applied to hypoxic conditions the breath holding method described by JONES *et al* (1958) for room air breathing In this method it is assumed that the partial pressure of CO in the alveolar air after breath holding should approximate the mean partial pressure of CO in pulmonary capillary blood

msec during sinus rhythm or following the first stimulus, following the second stimulus this takes up to 40–50 msec

In a fresh myocardial infarction conduction is still slower and paired stimulation invariably initiates ventricular fibrillation probably by re entry

This prohibits clinical application of paired stimulation in cardiogenic shock associated with acute myocardial infarction

W F H Stroer, *Changes in plasma conductance after administration of infusion fluids*

Pharmacological Dept of Proute Products N V, Amsterdam

The determination of the intravascular plasma volume with the aid of Evans' blue dye (T 1824) in rabbits after administration of 10 ml/kilo of infusion fluids is not always reliable It was thought that the conductance (C) of the plasma and its reciprocal value the resistance (R) might produce a valuable control Therefore the latter was determined in mixtures of plasma with different percentages of these fluids and curves showing the relation between the latter and R were constructed The agreement between the observed R values and those calculated by means of the law of Ohm was remarkable

According to this law

$$\frac{1}{R} = \frac{1}{\frac{a_1+a_2}{a_1}R_1} + \frac{1}{\frac{a_1+a_2}{a_2}R_2}$$

a_1 and a_2 are the volumes of the fluids with resistances R_1 and R_2 These curves can also be used to determine the percentage glucose or dextran glucose of a fluid when its resistance is known as was shown in three examples

1) In a rabbit 10 min after administration of a 5 % glucose solution, two thirds of the glucose are resorbed At that moment the glucose percentage of the plasma determined by a corrected Hagedorn Jensen method agrees exactly with that looked up in the afore mentioned diagram with the aid of the R calculated by applying Ohm s law to the observed resistances

2 and 3) Comparable results were obtained in two other rabbits after injecting 10 % dextran 40 000 (Rheomacrodex) or

inside of the mouth with 3 % borax in glycerol or with a nystatine paste freshly made by grinding one tablet of 500 000 I U in a mortar with one ml of glycerol) The patches disappeared, tumours were shed and the mucous membranes healed in a few days especially by using nystatine

The infection recurred however, although no other cases appeared after we removed all the sand from the terraria of our Boidae Close inspection of the mouth revealed the teeth to be the source of the recurrent infection Therefore an aggressive fungicidal treatment was started Once a week all visibly infected teeth were broken out, after which the inside of the mouth was coated with nystatine The treatment was completed in 6 and in 2 sessions respectively, in which a total number of 27 and of 7 teeth were removed No additional treatment was given All lost teeth were replaced by newly grown ones and the condition of both snakes is still excellent one year since their cure

J W Viersma L N Bouman and M Mater, *The influence of ouabain on conduction velocity and rapid depolarization in rabbit auricles at different frequencies*

Department of Physiology University of Amsterdam

In the crista terminalis of isolated rabbit atria we observed a relation between stimulation frequency and conduction velocity as well as a relation between stimulation frequency and maximal rate of rise of the transmembrane potential (VIERSMA *et al*, 1968) The muscle strip was driven with a programmed series of stimuli, the frequencies of which were stepwise changed between 2 and 9 cps After adaptation to the new frequency a steady state occurred The mean values of 20 measurements during a steady state were compared Both conduction velocity and maximal rate of rise of the action potential decreased exponentially at an increase in frequency

According to TRAUTWEIN (1963) a decrease in conduction velocity in the heart might be due to a decrease in the maximal rate of rise The latter depends on the sodium influx at depolarization There is evidence that a diminished sodium concentration gradient across the cell membrane will result in a decrease of the maximal rate of rise of the action potential Our hypothesis is that a relative

The values of $P_{c_{CO}}$ obtained after 6, 11, 16, 21, and 26 sec of breath-holding following breathing a hypoxic mixture were compared with the values of $P_{c_{CO}}$ estimated according to FORSTER *et al* (1957) in seven healthy male supine subjects The Forster method with pure oxygen breathing always preceded breathing the low oxygen mixture and breath-holding in the same subject

In all cases but one the value of $P_{c_{CO}}$ was maximal after 11 sec of breath-holding The values of $P_{c_{CO}}$ in the alveolar air after 11 sec of breath-holding were, on the average, 0 0034 mm Hg higher than those according to FORSTER *et al*, with S E = 0 0012 Since *no* CO was inhaled between the rebreathing and breath-holding periods, all CO in the alveolar air after breath-holding must have originated from the pulmonary capillary blood Consequently the higher values of CO pressure found after 11 sec of breath-holding should be a better approximation of the mean real $P_{c_{CO}}$ than those calculated according to FORSTER *et al* From a practical point of view the difference between these two methods is negligible, however, if we consider its manifestation in the calculated value of $D_{L_{CO}}$ But the breath-holding method has the advantage of being more simple and of not involving the complication of breathing a high oxygen mixture

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A A Verveen and M Kooy, *Two successfully treated cases of mouth-rot in giant snakes*

Department of Physiology, University of Leiden

Two Boidae of our collection, a *Constrictor constrictor* and an *Epicrates cenchris* Columbia, developed mouth-rot after devouring prey covered with humid sand The mucous membranes were densely covered with yellowish patches, there was a tumour of a few cm length in a corner of the mouth and several teeth were loose The infection, probably caused by a fungus, only partly responded to non aggressive fungicidal treatment (covering the

inside of the mouth with 3 % borax in glycerol or with a nystatine paste, freshly made by grinding one tablet of 500 000 I U in a mortar with one ml of glycerol) The patches disappeared, tumours were shed and the mucous membranes healed in a few days especially by using nystatine

The infection recurred, however, although no other cases appeared after we removed all the sand from the terraria of our Boidae Close inspection of the mouth revealed the teeth to be the source of the recurrent infection Therefore an aggressive fungicidal treatment was started Once a week all visibly infected teeth were broken out, after which the inside of the mouth was coated with nystatine The treatment was completed in 6 and in 2 sessions respectively, in which a total number of 27 and of 7 teeth were removed No additional treatment was given All lost teeth were replaced by newly grown ones and the condition of both snakes is still excellent, one year since their cure

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insufficiency of the sodium potassium pump at higher frequencies of stimulation causes a reduction of the concentration gradient of sodium ions across the cell membrane

By adding ouabain (strophantin G) to the perfusion fluid in a concentration of 10^{-7} g/ml, the sodium potassium pump was selectively inhibited (SKOU, 1965) After 15 min of equilibration another series of stepwise changing frequencies was started in order to compare the influence of frequency during ouabain administration with the original frequency effect

In all 15 experiments we observed during ouabain administration an increase of the influence of frequency on the maximal rate of rise as well as on the conduction velocity in the atrium

We managed to record intracellularly from one fibre for more than 60 min 20 min after the removal of ouabain from the normal perfusion fluid the influence of stimulation frequency on the mentioned parameters was restored to the original values

These results are in good agreement with the hypothesis that an increase in frequency involves an increase of the intracellular sodium concentration A decrease in the sodium concentration gradient across the cell membrane causes a smaller maximal rate of rise of the action potential and consequently a smaller conduction velocity along the atrium

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VIERSMA, J W, L N BOUMAN and M MATER, Nature 217, 1176 (1968)
TRAUTWEIN, W, *Pharmacol Rev* 15, 277 (1963)
SKOU, J C, Physiol Rev 45, 596 (1965)

J. Vos, K Kuriyama and E Roberts, *Transmitter binding in relation to the presence of acid mucopolysaccharides in subcellular fractions of mouse brain*

Netherlands Central Institute for Brain Research, Amsterdam, and City of Hope Medical Center, Duarte, Calif U S A

The binding of transmitter substances to nerve endings (Ne) and synaptic vesicles (SV) is supposed to be one of the initial steps of transmitter storage in SV before it can be released by action potentials

The *in vitro* binding of transmitter substances showed some characteristics of a cation exchange process (KURIYAMA, ROBERTS

and Vos, 1968) For this reason the electrokinetic properties of Ne and SV were studied by means of electrophoresis in a sucrose gradient It was found that Ne and SV have a net negative surface charge at pH7 and an isoelectric point at approximately pH4

This negative surface charge was not related to sialic acid as is the case in several cell types The binding of transmitter substances showed no clear relationship between surface charge and binding, *e g* about 2 × more serotonin was bound per mg protein than norepinephrine or acetylcholine, while more acetylcholine was bound to SV than to Ne (Vos, KURIYAMA and ROBERTS, 1968)

In an attempt to identify the nature of the anionic groups involved in the surface charge and the cation exchange phenomena, attention was focussed on polyanions such as acid mucopolysaccharides (AMPS) It was found that whole mouse brain contained 0 02 % AMPS and about 60 % sediments with the particulate fraction of a homogenate at *100 000 g* After extraction with chloroform methanol 2 1 and drying, the lipid free dry material (LFDM) of the particulate fraction of whole mouse brains contained 1 13 mg AMPS per g of LFDM, while synaptic vesicles contained 2 31 mg AMPS per g of LFDM, nerve endings 0 61 mg AMPS per g LFDM, and brain mitochondria only 0 23 mg AMPS per g LFDM

The AMPS in the different fractions were identified as chondroitin sulfate (95 % in whole brain) and hyaluronic acid A trace amount of unidentified AMPS was present in all fractions

The presence of the relatively large amount of AMPS in SV is of particular importance with regard to binding studies A possible role of AMPS in the binding and release of transmitter substances *in vivo* cannot be excluded nor confirmed at the moment

F A de Wolff H van Rees L I Swaab and E L Noach,
"Indirect" sympathicomimetics and the isolated seminal vesicle

Dept of Obstetrics and Gynaecology University Hospital, Leiden
Dept of Pharmacology, University of Leiden

From clinical experience it is known that hypertensive patients, treated with guanethidine, often suffer from ejaculation disturbances Data from the literature indicate a sympathetic innervation of the prostate, seminal vesicle and ductus deferens

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From clinical experience it is known that hypertensive patients, treated with guanethidine, often suffer from ejaculation disturbances Data from the literature indicate a sympathetic innervation of the prostate seminal vesicle and ductus deferens

Using the isolated seminal vesicle of the rat as test organ (according to LEITCH *et al*, 1954), it was investigated whether guanethidine decreases the contractility of these accessory male sex organs via depletion of the adrenergic neurotransmitter

In analogy to blood pressure experiments according to BURN and RAND (1958) it was examined whether the sympathicomimetic effect of tyramine on the contractions of the seminal vesicles is modified by guanethidine Contrary to expectations, however, tyramine did not cause any contractions, it even sometimes reduced contractions brought about by noradrenaline This inhibition cannot be interpreted as a β mimetic effect, since isuprel did not affect the contractions of the seminal vesicle

Pretreatment of rats with 100 mg/kg/day guanethidine intraperitoneally during 7–12 days did not lead to any significant sensitization to adrenaline or noradrenaline, although the noradrenaline content of the seminal vesicles after pretreatment was reduced to zero

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J W DUYFF, 1907-1969

IN MEMORIAM PROFESSOR J W DUYFF

On October 6th died, at the age of 62, Jan Willem Duyff, professor of physiology at the University of Leiden, founder and editor in chief of these Acta

He started his scientific career already as a medical student at the University of Amsterdam where he worked at the department of histology He graduated in 1931, practised general medicine for some time and became instructor of histology in 1932 at the *University of Amsterdam*

His early work is concerned with lipid metabolism and with problems of growth, cell differentiation and the mutual interaction of growing tissues This led him to the study of electrical properties of living tissue and to bio-electricity In 1942 he passed his thesis on 'Low frequency impedance of animal tissue"

During the war he participated actively in the armed resistance against the German occupation and became one of the leaders of the Dutch Underground Forces

For his resistance work he was rewarded with the Medal of Freedom with Bronze Palm (U S A) and was appointed as an Honorary Member in the Military Division of the Order of the British Empire and Officier in the Dutch Order of Oranje Nassau with Swords Later he became a Knight in the Order of the Nederlandse Leeuw In 1946 he was appointed professor of physiology at the University of Leiden

In the early years of his professorship he designed and created the new physiological laboratory, the center of the XXII International Congress of Physiological Sciences in 1962, which he organised almost single handedly and of which he was the Congress President

His many activities gave a new impetus to a general uplift of Dutch physiology, in particular at Leiden Among these was the foundation of the Acta Physiologica et Pharmacologica Neerlandica in 1950 of which he was the very critical chairman of the editorial board The number of his own papers during his professorship is limited but he initiated and stimulated many and his critical approach and vast knowledge of the sciences and humaniora put a mark on the papers of his pupils

He supervised some 15 theses, most of which appeared as

preprints of these Acta That in itself was a novelty in Dutch academic traditions

From 1956 onward he also was an active member of the editorial board of the Dutch Medical Journal (Ned Tijdschrift voor Geneeskunde) He was a fervent advocate of the introduction of computers in medical and scientific data processing and the main part of his scientific activities for the past 10–15 years was devoted to this field

He held many offices in the Dutch and international scientific world He is probably best known abroad as delegate to the I U P S of which he was a member of the Council since 1959, its Vice President since 1962 and recently its Secretary He was a man of strong character and a tremendous energy who never stopped working, in spite of his being seriously ill for the past two years His impact on physiology and the whole field of medical sciences in the Netherlands has been great, although his natural modesty led him to remain in the background as much as possible

P E Voorhoeve

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Department of Physiology University of Leyden

THE TRANSFER FUNCTION OF FROG MYONEURAL JUNCTIONS

II THE INFLUENCE OF PREVIOUS CONDITIONS *IN SITU* UPON THE *IN VITRO* RESPONSES TO PULSE TRAINS

BY

A C BOBBERT

CONTENTS

1 INTRODUCTION, PLAN OF INVESTIGATION

From the results of a previous investigation (BOBBERT, 1969) it was concluded that during repetitive indirect stimulation of isolated frog muscles there occurs a progressive lowering of the response/stimulus ratio of the twitch fibres which results both from fatigue and ageing of the preparations In these experiments the impression arose that the rate of decrease of this ratio depended on the lapse of time between sectioning of the nerve and interruption of the blood supply to the muscle

To establish this definitely, experiments were made which also served to ascertain whether the two types of motor units present in the frog's twitch system i e the units in which the response to the second pulse of a twin is facilitated and those in which it is depressed (BOBBERT, l c), behave in a consistent way in response to the third and later pulses of pulse trains

2 MATERIALS AND METHODS

These were virtually the same as in the experiments reported earlier

2 1 BASIC PROCEDURES

The preparations, which always consisted of the sciatic nerve and gastrocnemius muscle of *Rana Temporaria* or *R Esculenta* of either sex and nearly the same size were usually examined *in vitro* in a bath of paraffin oil at a temperature between 18 and 21 °C

The results of the experiments were independent of the animal's sex and not appreciably influenced by the season

2 2 STIMULI were applied to the nerve or directly to the muscle They consisted of rectangular 1 msec pulses of equal voltage applied at the rate of 1 per 10 sec Any of these single pulses could be replaced by a train of up to five pulses with adjustable intervals

2 3 RECORDING

Mechanical records were obtained with a conventional isotonic lever and kymograph

Electrical records were led off between an active electrode piercing the *tendo calcanei* and an earthed electrode The potential changes

were amplified 1000 × by means of a pre amplifier the low pass filter of which was set at an R-C time of 2 msec and photographed as single shots from the oscilloscope screen

2 4 SEQUENCE OF EVENTS DURING A TYPICAL EXPERIMENT

In most experiments the pulses were supramaximal Prior to the experiment proper 1 msec test pulses of stepwise increasing voltage were given to determine the "maximal stimulus voltage for single pulses of this duration the number of these test pulses never exceeded seven In the experiments themselves stimulus strength was at least 115 pct of the just maximal pulse intensity One stimulus pulse was applied every 10 sec, except that every 30th stimulus consisted of a train of five pulses at intervals of 25 msec unless indicated otherwise Records were made of the electrical responses to these trains (mV) and of the mechanical responses to the trains and to the immediately preceding single pulses (arbitrary units)

To obtain data suitable for comparing the results of different types of experiment the response amplitudes of four preparations were averaged and represented as such unless otherwise indicated

3 RESULTS OF STIMULATION WITH SUPRA-MAXIMAL PULSES

3 1 CHANGES IN RESPONSE IN THE COURSE OF REPETITIVE STIMULATION

3 1 1 *Electrical and mechanical responses during indirect stimulation*

In four decerebrate frogs breathing spontaneously and with an intact circulation both sciatic nerves were sectioned and carefully prepared free from the surrounding tissues without causing undue loss of blood or impairment of the local circulation Then within 15 min after denervation the blood supply to one leg was blocked, after which the frog was mounted with its legs bathing in paraffin-oil

An experiment was made with the so obtained 'E (early) preparation Upon completion of this experiment i e about 150–180 min after denervation the circulation through the other leg was interrupted and an identical experiment was made with the "L- (late) preparation thus obtained

The essential difference between these two types of preparation lies in the fact that before the experiment proper, sectioning of the nerves was performed in the *L* preparations at 150–180 min before the blood supply was interrupted which *gives the local circulation ample opportunity to* neutralize possible effects resulting from the previous physiological nervous inflow while this opportunity is highly restricted in the case of *E* preparations

Part of the data obtained in the experiments with supramaximal stimulation of *E* and *L*-preparations are given in Figs 1 and 2 In Fig 1 the changes in amplitude of the electromyograms due to the first and to the fifth pulses of the trains are plotted against the number of applied pulses for both the *E* and the *L*-preparations

In Fig 2 the same has been done for the amplitudes of the mechanical responses to single pulses and to trains of five pulses at intervals of 25 msec It appears from Fig 1 that during repetitive indirect stimulation the electrical responses to the first and to the fifth pulses of the trains diminish strongly for both types of preparation but that the rate of this decrease is considerably lower for the *L*-preparations This difference is still more marked in the case of the mechanogram amplitudes (Fig 2) at the time when in the *E* preparations the mechanical responses to single pulses and even to pulse trains have disappeared they may have decreased by only 70 pct in the *L* preparations

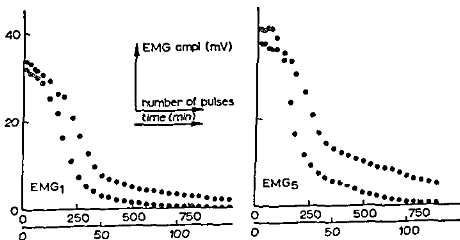


Fig 1
Changes in amplitude of the electrical responses to the first (left) and fifth (right) pulses of trains during indirect stimulation of *E* (◉) and *L* (●) preparations

These observations naturally raised the question whether the differences between E and L-preparations are linked with synaptic or presynaptic phenomena, or result from an influence of denerva tion in the presence of an intact blood supply, upon the post-synaptic elements For this reason similar experiments were made with *direct* stimulation

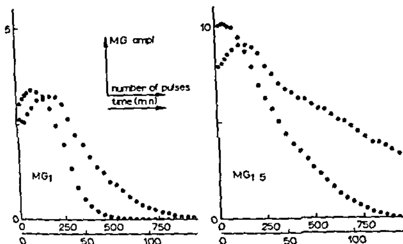


Fig 2
Changes in amplitude of the mechanical responses to single pulses (left) and to trains of 5 pulses (right) during indirect stimulation of E (◉) and L (●) preparations (Note the different vertical scales)

3 1 2 *Mechanical responses during direct stimulation*

In four decerebrate frogs one sciatic nerve was sectioned 10 min after an intraperitoneal injection of 80 mg Flaxédil Then, about 15 min after denervation, the circulation on that side was blocked and the resulting "E preparation' was mounted for direct stimu lation In another four decerebrate preparations one sciatic nerve was sectioned and the operation field bathed in paraffin oil for 130–150 min after which the frog was paralyzed by means of an identical Flaxedil injection Twenty five minutes later, i e again at 150–180 min after sectioning of the nerve, the local blood supply was blocked on the denervated side and the resulting "L-prepara tion" was stimulated directly according to the usual pattern

Because the stimulus artifacts resulting from the high voltage pulses needed for direct stimulation of all muscle fibres largely obscured the electrical responses of the muscles, only the mechanograms could be used (BOBBERT, *l c*)

In Fig 3 the changes in amplitude of the directly evoked mechanograms in response to single pulses and to the pulse trains are plotted against the number of preceding pulses, for both the *E*- and the *L* preparations It appears that, here again, there is a marked staircase effect in the responses to single pulses which is less pronounced or even absent, in the responses to the pulse trains Like in previous experiments the staircase period is attended by an increase in resting length of the muscles (not shown) The staircase is more pronounced and of longer duration in the *L* preparations than in those in which the circulation was interrupted shortly after denervation (Fig 3) Because the same differences were observed for *E* and *L* preparations stimulated *via* the nerves (Fig 2), it is obvious that they result from an influence of denervation, long before blocking of the local blood supply, upon the muscle fibres themselves

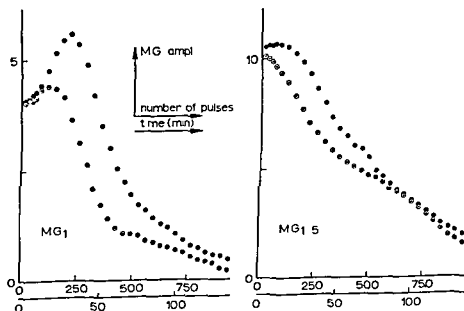


Fig 3

Changes in amplitude of the mechanograms in response to single pulses (left) and to trains of 5 pulses (right) during direct stimulation of *E* (◉) and *L* (●) preparations

After the staircase period the amplitudes of the mechanical responses of the E and the L-preparations decrease at approximately the same rate, in contrast with their behaviour during indirect stimulation (Fig 2) This suggests that the difference between the rates of decrease of the mechanical responses of the E and the L-preparations during *indirect* stimulation is closely connected with the different rates of decrease of their electrical responses (Fig 1) and that both result from a slower decrease of the response/stimulus ratio of the myoneural junctions in L-preparations

If this should be the case, then it is to be expected that the facilitatory effect of conditioning pulses upon the responses to test pulses as expressed in the ratio between the amplitudes of the electromyograms and that between the mechanograms (BOBBERT, l.c.) will be more pronounced for the indirectly stimulated E-preparations than for the L preparations and that the difference will increase in the course of stimulation

3 1 3 *Comparison between the mechanogram amplitude ratios during direct and indirect stimulation*

In Fig 4 the ratio between the amplitudes of the mechanical responses of E and L-preparations to single pulses and to pulse trains is plotted against the number of preceding pulses, for direct and for indirect stimulation In the case of *direct* stimulation (left) the MG amplitude ratios of the two types of preparation are nearly identical and decrease slowly, during the staircase period After that both show a gradual increase that mainly results from the inertia of the isotonic lever, which favours recording of the contractions due to pulse trains above that of the weaker responses to single pulses This also results in a difference between the MG amplitude ratios of E and L-preparations after the staircase period, the ratio for the L-preparations being lower because their mechanical [illegible] staircase [illegible] 4, right) [illegible] amplitude ratios are higher during the staircase period than in the case of direct stimulation although, again, they decrease slightly and are virtually identical But now, after this period they increase more rapidly than during direct stimulation, this steep increase is especially marked for the E preparations These changes

in the MG amplitude ratio during indirect stimulation more or less correspond to the simultaneously occurring changes in the ratio between the amplitudes of the electrical responses to the fifth and first pulses of the trains (Fig. 5). Therefore, it appears that in the course of indirect stimulation the response/stimulus ratio of the myoneural junctions decreases more slowly in the *L*-preparations than in the *E*-preparations, which explains the differences between the rates of decrease in amplitude of their electrical (Fig. 1) and mechanical (Fig. 2) responses. As a result, the facilitatory effect of conditioning pulses will express itself more clearly in the *E*-preparations, which results in a larger increase of the amplitude ratio of the electrical responses to the fifth and first pulses of the trains (Fig. 5) and, as a consequence, of that between the mechanical responses to pulse trains and to single pulses (Fig. 4).

This poses the question whether the increasing difference in response/stimulus ratio between the neuromuscular junctions of *E*- and of *L*-preparations in the course of 1 per 10 sec stimulation

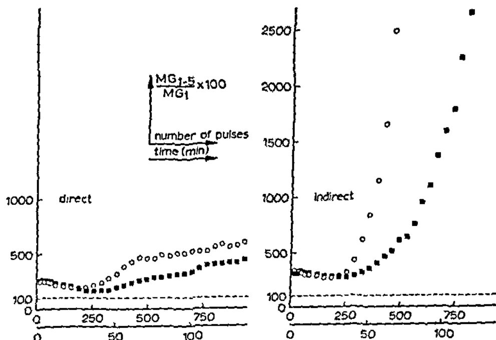


Fig. 4.

Changes in the ratio between the amplitudes of the mechanical responses to trains of 5 pulses and of those to single pulses during direct (left) and indirect (right) stimulation of *E*- (○) and *L*- (■) preparations.

is due to the increase, in time, of the number of stimuli applied, or to deterioration In order to answer this question ageing experiments were made

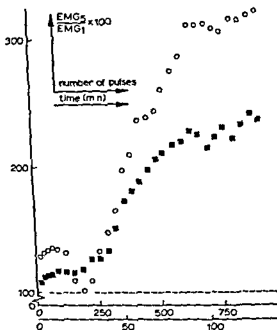


Fig 5

Changes in the ratio between the amplitudes of the electromyograms in response to the fifth and to the first pulses of trains during indirect stimulation of *E* (○) and *L* (■) preparations

3 2 Changes in response due to ageing of in *vitro* preparations

In the same way as described in section 3 1 1 "*E*" and "*L*" preparations were obtained from 4 frogs In these preparations the influence of ageing on their responses to indirect stimulation according to the standard pattern (2 4) was investigated in experiments lasting for 3 h test stimuli only being applied at 30 min intervals These test stimuli consisted of a supramaximal single pulse followed, after 10 sec, by a train of five of such pulses delivered at 25 msec intervals

In Fig 6 the normalized changes, due to ageing, in the amplitudes of the electrical responses to the first pulses of indirectly applied pulse trains are shown for the E and the L preparations, together with those observed in the experiments with indirect stimulation at the rate of 1 per 10 sec (3 1 1, Fig 1) From the ageing experiments *it appears that, after a slight staircase effect in the responses* to single pulses (which did not appear during repetitive stimulation and whose nature, therefore, was not investigated), the electrical responses decrease in amplitude for both types of preparation However, the rate of decrease is much lower in the case of the L preparations, the amplitude after 120 min of deterioration being virtually unchanged in the L preparations against its being reduced by more than 50 pct in the others If these data are compared with the corresponding values in the course of repetitive stimulation (*93 and practically 100 pct, respectively*), *it becomes evident that* the increasing difference in response/stimulus ratio between the

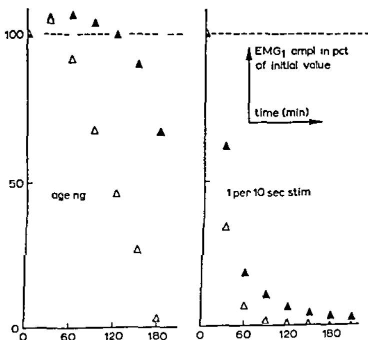


Fig 6

Normalized changes in the average amplitudes of the electrical responses of E (△) and L (▲) preparations to indirectly applied single pulses occurring as a result of ageing (left) and during 1 per *10 sec stimulation* (right)

two types of preparation in the course of 1 per 10 sec stimulation (Fig 1) largely results from a difference of the speed at which they deteriorate

In order to elucidate in which way, after blocking of the nervous inflow, the state of the local circulation influences the transfer at myoneural junctions the ratio between the amplitudes of the electrical responses to the fifth and first pulses of the trains, which gives an impression about the degree to which the response/stimulus ratio of motor endplates can be raised by conditioning (BOBBERT, *l c*) was calculated for both types of preparation In Fig 7 these ratios are plotted against time both for the ageing experiments and for those with repetitive stimulation From the ageing experiments it appears that for the *L*-preparations the ratio is fairly high initially and is *not* significantly influenced by ageing, while in the *E* preparations there is a slight decrease during the

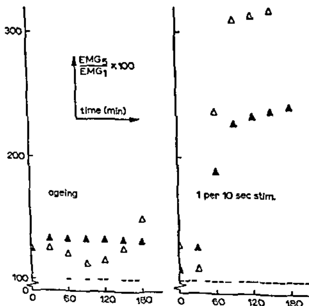


Fig 7

Changes in the ratio between the amplitudes of the electromyograms in response to the fifth and to the first pulses of trains indirectly applied to *E* (Δ) and *L* (▲) preparations in ageing experiments (left) and during 1 per 10 sec stimulation (right)

first 90 min and a fairly steep increase afterwards If the behaviour of the ratio in the course of ageing is compared with that during *repetitive stimulation* (Fig 7, right), it becomes evident that there is little correspondence between the influence on the transfer function, of the relation between the *state of the circulation* and that of the nervous inflow prior to the experiments proper, in the ageing preparations and in those stimulated at the rate of 1 per 10 sec

As mentioned before, both in the ageing experiments and in those with repetitive stimulation, the amplitudes of the electro myograms decrease for *L* and *E* preparations, while in both types of experiment the rate of this decrease is higher in the case of *E* preparations (Fig 6) Now, if this difference between the *L* and *E* preparations would result from the *same* effect of the state of the circulation prior to the experiments, exerted at the same *point in the chain of events leading from the application of a* supraliminal stimulus to a motor axon to the generation of an action potential by the twitch fibres it subserves, there can be no difference between the effects of conditioning pulses in the ageing experiments and in those with repetitive stimulation Because such a difference exists (Fig 7) it may then be concluded that the way in which the circulation, if left intact for hours after blocking of the nervous inflow, acts to prevent a decrease, due to ageing, in EMG amplitude is *not* the same in which it does so in the case of repetitive stimulation

Fom the results of previous experiments (BOBBERT, *l c*) it was concluded that, in the case of stimulation at the rate of 1 per 10 sec the fatigue of neuromuscular transmission has a fairly large synaptic component char acterized by a decline in the number of acetylcholine quanta released per impulse and as a consequence *a decrease in the response/stimulus ratio* of the muscle fibres In those experiments it was further shown that in many *motor units* of the frog s twitch system the decrease in quantum content of the EPP s can be partly offset by the facilitatory after effect of a con ditioning impulse as evidenced by an increased response/stimulus ratio of single muscle fibres *for a closely following impulse and by an increase in* the ratio between the electrical overall responses of the muscle to the second and first pulses of twins

In the present experiments similar observations were made as to the relation between the amplitudes of the EMG's due to the second and first pulses of the pulse trains (not shown) and for that

between the amplitudes of the EMG s in response to the fifth and first pulses of these trains (Figs 5 and 7) The difference observed in these experiments between the rates of decrease of the EMG amplitudes in *L*- and *E* preparations (Fig 1, and right half of Fig 6) and those between the changes in their EMG amplitude ratios (Fig 5 and right half of Fig 7) can easily be explained on the reasonable assumption that the *L*-preparations have *larger stores* of readily releasable acetylcholine than the *E* preparations In the intact frog these stores are then partly depleted as a result of a relative insufficiency of the blood supply to cope with the continuous drain on these stores exerted by the physiological inflow of impulses to the myoneural junctions and they are replenished in the *L*-preparations during the long period in which there was an intact blood supply and no nervous inflow The initially larger number of transmitter quanta stored in the *L* preparations would result in a less rapid decrease of the transmitter output per impulse and therefore in a smaller difference between the responses to conditioning pulses and to test pulses

Incidentally it may be remarked that this effect of the circulation on the difference between the responses to single pulses and to pulse trains or twins will hardly play any role in classroom experiments on summation of contractions during ind rect stimulation (cf BOBBERT *l c* sect on 1) because in preparations used for such experiments the c rculation has usually been interrupted at the same moment as the nervous inflow

For the case of age ng preparations it was concluded from previous experiments on direct and ind rect activat on of frog gastrocnemii that the decreases in amplitude of the electrical and mechanical responses to single pulses and to twins wh ch occur during the first three hours and are not accompanied by an increase in the ratio between the amplitudes of the elec trical responses to the second and first pulses of twins are mainly due to a deterioration of the postsynaptic elements

Similar observations were made in the present ageing experiments with regard to the decrease in amplitude of the electrical response to the first pulse of trains (Fig 6 left) which is not accompanied by a sizable increase in the ratio between the amplitudes *of the* electrical responses to the fifth and those to the first pulses of the trains (Fig 7 left) This raised the question whether the differences between the changes in amplitude of the EMG s of the *L* and the *E* preparations (left halves of Figs 6 and 7) *also result* from

a more rapid deterioration of the postsynaptic elements in the *E*-preparations. This was investigated in *L*- and *E*-preparations obtained from 8 frogs in the manner described in section 3 1.2., in which the effect of ageing on their mechanical responses to *direct* activation was examined with test stimuli applied every 30 min. These test stimuli consisted of a single pulse, followed after 10 sec by a train of 5 of such pulses delivered at 25 msec intervals. The results of these experiments are shown in Fig. 8 where the normalized changes in the amplitudes of the mechanical responses to the pulse trains are plotted against time, together with those observed in the previously described ageing experiments in which the test stimuli were applied *indirectly*. From the left half of this figure it appears that there is hardly any difference in the rates of decrease of directly evoked mechanical responses between ageing *L*- and *E*-preparations, although there is a large difference between the rates

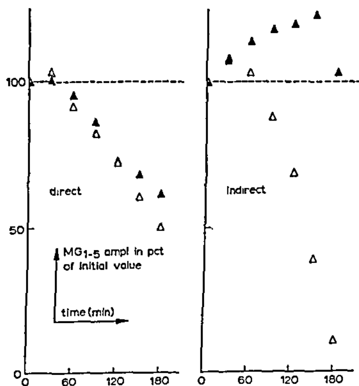


Fig. 8.

Normalized changes in the average amplitude of the mechanical responses to directly (left) and indirectly (right) applied trains of 5 pulses, occurring in ageing *E*- (Δ) and *L*- (▲) preparations

of decrease of their electrical (Fig 6, left) and mechanical (Fig 8, right) responses to indirectly applied test stimuli

Therefore, it may be concluded that the marked difference in rate of decrease of the EMG amplitudes between ageing *L*- and *E*-preparations (Fig 6 left) does not result from a difference in the rate at which their muscle fibres deteriorate but is due to a difference in condition of the presynaptic elements

The implied protective action on these elements, exerted by an intact circulation, might be due to

(a) prevention of deterioration and depolarization of the membranes around the terminal endings of the motor axons This possibility is largely ruled out by the fact that in ageing experiments, and even during low frequency stimulation, there is, neither in the *L*- nor in the *E* preparations a significant decrease in amplitude of the electrical responses of the nerves to supramaximal pulses

Moreover, the electrical responses of the nerves to submaximal test pulses *increase* in the same way in both types of preparation (not illustrated)

(b) an increase in the amounts of readily releasable acetyl choline As mentioned before, the existence of this effect can be derived from the difference in behaviour between the electromyograms of *L* and *E* preparations in the course of repetitive indirect stimulation (Fig 6 right) and from the concomitant more rapid increase of the EMG ratio in the case of *E* preparations (Fig 7 right) Because such changes in the EMG ratio hardly occur during ageing [illegible]

L and *E* preparations must largely result from another effect of the circulation

(c) prevention of deterioration of the mechanism coupling presynaptic depolarization and acetylcholine release It is highly probable that deterioration of this mechanism results in a decrease of the postsynaptic responses without a sizable change in the ratio between the amplitudes of the EMG's due to test pulses and to conditioning pulses On the assumption that a period when the nervous inflow to the junctions has been stopped while their blood supply has been left intact lowers the rate at which this coupling

mechanism deteriorates the difference in ageing experiments between the changes in the response amplitudes of indirectly activated *L* and *E* preparations (left halves of Figs 6 and 7, right part of Fig 8) is easily explained

In summary it may be stated that *L* preparations differ from *F preparations in at least two ways* in larger stores of acetyl choline and in a slower deterioration of the mechanism for de polarization-release coupling *It is obvious that both result in a* slower decrease of the response/stimulus ratio in the *L* preparations during long continued indirect stimulation (Fig 6 right)

The results of earlier experiments on indirect stimulation of frog muscles with single pulses and twins yielded strong evidence for the presence in the frog s twitch system of two types of motor *units one with a facilitatory and the other with an inhibitory* response to a conditioning pulse A subdivision of the twitch units *along this line would have a firmer basis if it were established that* the units of either type show a similar effect of conditioning in their responses to the third and later pulses of pulse trains as in those to the second pulses This was analyzed in experiments in which the strength of the test stimuli was varied

4 RESULTS OF INDIRECT STIMULATION WITH PULSES OF VARIED VOLTAGE

Four preparations were subjected to three series of indirect stimuli administered according to a pattern in which during stimulation at the rate of 1 per 10 sec every sixth pulse was replaced by a train of five pulses at 25 msec intervals During application of each series the voltage range from supramaximal to subliminal was covered *in 20 steps at one minute intervals*

The first series was applied when the blood supply was still *intact while the nerve had been sectioned* the second and third series were started at 40 and at 90 min respectively after inter ruption of the circulation In the periods between these test runs supramaximal stimuli were applied at the usual rate in order to fatigue the preparations

Because apart from quantitative differences the results of these four experiments were substantially the same only those obtained with one of the preparations are given in Fig 9 This figure shows

the manner in which the amplitude of the EMG in response to single pulses varies with their strength, together with the ratios between the amplitudes of the EMG's due to the second to fifth pulses of the trains and of those resulting from their first pulses From graph I it appears that, when the blood supply to the muscle is still intact, the amplitude of the EMG in response to single pulses varies with their strength according to the usual *S* shaped curve It further appears that the EMG ratios are above unity over the whole range of pulse strengths except for a rather narrow range

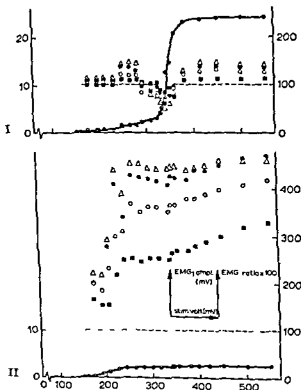


Fig 9

Plots, against stimulus strength, of the amplitudes of the electromyograms of a muscle elicited by the first pulses of indirectly applied pulse trains (◉), together with the amplitude ratios EMG_2/EMG_1 (■), EMG_3/EMG_1 (○), EMG_4/EMG_1 (●) and EMG_5/EMG_1 (△) The curves are derived from data obtained when the blood supply was intact (I) and during a 20 min period starting at 90 min after interruption of the circulation (II)

mechanism deteriorates, the difference, in ageing experiments, between the changes in the response amplitudes of indirectly activated L and E preparations (left halves of Figs 6 and 7, right part of Fig 8) is easily explained

In summary it may be stated that L preparations differ from E preparations in at least two ways *viz* in larger stores of acetyl choline *and in a slower deterioration* of the mechanism for de polarization–release coupling It is obvious that both result in a slower decrease of the response/stimulus ratio in the L preparations during long continued indirect stimulation (Fig 6, right)

The results of earlier experiments on indirect stimulation of frog muscles with single pulses and twins yielded strong evidence for the presence in the frog's twitch system, of two types of motor units, one with a facilitatory, and the other with an inhibitory response to a conditioning pulse A subdivision of the twitch units along this line would have a firmer basis if it were established that the units of either type show a similar effect of conditioning in their responses to the third and later pulses of pulse trains as in those to the second pulses This was analyzed in experiments in which the strength of the test stimuli was varied

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Because, apart from quantitative differences, the results of these four experiments were substantially the same only those obtained with one of the preparations are given in Fig 9 This figure shows

This is in accordance with previous observations on the change in the responses to twin pulses in the course of stimulation at the same rate

While graph I of Fig 9 shows that for each type of motor unit, the conditioning action of a train of pulses spaced at 25 msec intervals increases with the number of pulses in the train it appears from graph II that the magnitude of its effect on the response/stimulus ratio of the units showing facilitation increases markedly with increase in pulse voltage of the trains If the latter fact is considered in connexion with the more rapid decrease in amplitude of the electrical responses to the pulses of higher strengths, it may be concluded that the speed at which fatigue of neuromuscular transmission develops in the units with a facilitatory response increases with the threshold for activation of their motor axons [1])

It is highly probable that this proportional relation between the speed at which fatigue of transmission develops at the junctions and the threshold of their axons is actually a much closer one than may be derived from Fig 9 because in this experiment the number of the impulses that have arrived at the junctions during administration of the series must have been inversely related to the threshold of their axons.

In view of the aforementioned conclusions, it is interesting to know whether the changes in the relative amplitudes of the EMG's due to the separate pulses of test trains, appearing in the course of indirect stimulation *in vitro* with supramaximal pulses, can be explained on the basis of these conclusions

5 INFLUENCE OF SUPRAMAXIMAL INDIRECT STIMULATION ON THE RESPONSES OF *IN VITRO* PREPARATIONS TO TRAINS OF PULSES AT EQUAL AND UNEQUAL INTERVALS

The data on the electrical responses of the *E* and *L*-preparations to trains of five supramaximal pulses at 25 msec intervals (*cf* section 3 1 1) were overall averaged The results are given in Fig 10 which shows plotted against the number of pulses applied, the changes in amplitude of the EMG's due to the first pulses of the

[1]) From Fig 9 it might be derived that with respect to this relation between the threshold of the axon and the rate at which fatigue of its motor endplates develops during repetitive activation *in vitro* there is no difference between the axons belonging to motor units showing conditioned depression and those of units with a facilitatory response

It is further seen that for trains of a pulse strength on either side of this range the EMG ratios increase in the order from that between EMG_2 and EMG_1 to that between EMG_5 and EMG_1, while there is a strong tendency to a reversal of this order in the case of trains with a strength inside this voltage range From this it is evident that the population of motor units having axons with intermediate thresholds contains a *high percentage of units whose junctions* respond to every additional conditioning pulse with a further *lowering* of the response/stimulus ratio and that the motor unit populations activated by pulse trains of higher or lower intensities consist mainly of units having junctions at which the response/stimulus ratio *increases* progressively with the serial number of the pulses in the trains

> These observations strongly support the earlier statement (BOBBERT, *l c*) that the frog's twitch system contains two types of motor unit in which a conditioning pulse has opposite after effects, and for which, now, it has been established that *this also applies to conditioning* pulse *trains*

Graph II of Fig 9 shows that, after the application *in vitro* of *about 750 pulses to the nerve* [1]*), the S shaped relation between the* amplitude of the EMG's due to single pulses and the voltage of these pulses is no longer apparent, evidently because the EMG's in response to pulses of the higher intensities have strongly diminished in amplitude while those due to pulses of lower strengths have slightly increased The decrease in amplitude of the responses to pulses of the higher strengths must be due to fatigue and ageing of the preparations (*3 1 , 3 2*), effects that are outweighed *in the* responses to the weaker pulses by an increase in the number of activated axons resulting from a progressive rise in the electrical excitability of nerve fibres in the course of such prolonged *in vitro* experiments (BOBBERT, *l c*)

From a comparison of Fig 9 I and 9 II it appears that during repetitive stimulation *in vitro* each of the EMG ratios increases for any voltage of the pulses and *that, after some 750 pulses have* been applied, there remains no indication of a contribution to the responses made by the units characterized by conditioned depression

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1) The data obtained during administration of the second series are not shown because they differ only quantitatively from those of the third series

This decrease is more marked in the case of the EMG_5/EMG_1 and EMG_4/EMG_1 ratios than in that of EMG_3/EMG_1. These changes in the ratios are accompanied by a very steep decrease in amplitude of the EMG's due to single pulses and may be explained by the more rapid occurrence of junctional fatigue in the twitch units with high threshold axons, which comprise few units showing conditioned depression (4.)

After the application of about 200 pulses the rate at which the EMG_1 amplitudes decrease slows down while there is a rapid rise of all four EMG ratios, the steepness of which rise diminishes in the order from the EMG_2/EMG_1 ratio to that between EMG_5 and EMG_1. These changes in the responses may be explained on the assumption that the motor units which have high threshold axons and are of the facilitatory type have now dropped out of the picture and that, therefore, these changes result from a lowering of the response/stimulus ratio in the remaining motor units which are of both types. When some 500 pulses have been applied the EMG_2/EMG_1 ratio starts to decline, while the other EMG ratios do not rise any further and there is a strong tendency for the ratios to revert to the initial order of rank. The cause of this may be that the responses are now dominated by the motor units whose junctions are the least susceptible to fatigue, which units have low threshold axons and junctions where conditioning impulses only have a weak facilitatory effect (4.)

It is evident that the results of these experiments, in which the condition of indirectly stimulated preparations was tested by the application of trains composed of supramaximal pulses at *equal* intervals of 25 msec. can be explained on the basis of conclusions drawn from the results of experiments in which the condition of the preparations was tested by means of pulse trains of varying strengths. This is not too surprising if it is realized that between these types of experiment there was a difference only in the voltage of the pulses in the test trains.

Therefore the question may be raised whether the changes occurring during repetitive stimulation at the usual rate, in the response to trains of supramaximal pulses following each other at *unequal intervals*, can also be explained on the basis of these conclusions.

trains, together with the simultaneously occurring changes in the ratios between the amplitudes of the EMG's in response to the second to fifth pulses of a train and the amplitude of the EMG due to its first pulse

It appears that at the start of stimulation *in vitro* with supra maximal fatiguing pulses and trains of test pulses the EMG ratios are larger than unity and increase in the order from EMG_2/EMG_1 to EMG_5/EMG_1, which means that the responses of the twitch units showing conditioned facilitation outweigh those of the units exhibiting depression

It further means that for part of the twitch units the response/stimulus ratio in the case of single pulses is initially lower than 1 1, a conclusion already drawn from the results of earlier experiments (BOBBERT, *l c*)

After the application of some 100 pulses a period follows in which the EMG_2/EMG_1 ratio increases while the other ratios decrease

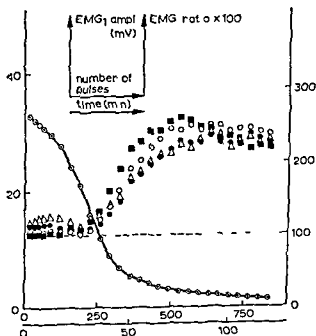


Fig 10 Changes in the average amplitudes of the electromyograms due to the first pulses of indirectly applied trains (◉) and of the amplitude ratios EMG_2/EMG_1 (■) EMG_3/EMG_1 (○) EMG_4/EMG_1 (●) and EMG_5/EMG_1 (△) in the course of indirect stimulation

spacing of the pulses in the trains, which results in a very strong conditioning effect on the responses to the third pulses and a weak one on those due to the fourth pulses After the application of about 100 pulses, during which there is a rather slow decrease in amplitude of the responses to single pulses, a period starts in which this amplitude decreases steeply In this period, again, there is a slight lowering of the EMG_2/EMG_1 ratio and even a drop below unity of the EMG_4/EMG_1 and EMG_5/EMG_1 ratios However, the EMG_3/EMG_1 ratio shows a very steep rise suggesting that, at 10 msec after application of the second pulse, the response/stimulus ratio is strongly raised in the units characterized by conditioned facilitation (while it must be markedly lowered in those of the other type) From the behaviour of the EMG_4/EMG_1 ratio it appears that, at 40 msec after application of the third pulses, the response/stimulus ratio of the units with a facilitatory response to conditioning pulses must be lower in the case of the fourth pulses of the trains than in that of the first pulses

If it is assumed that the marked rise of the EMG_3/EMG_1 ratio results from an augmented release of acetylcholine quanta per impulse in the motor units showing conditioned facilitation, then the decrease in amplitude of EMG_4 below that of EMG_1 might be explained by a partial depletion of the stores of readily releasable ACh when the fourth volley of impulses arrives by a temporary lowering in the number of ACh receptors that are unoccupied at this moment or by both However, from the fact that the EMG_5/EMG_1 ratio is slightly higher than that between EMG_4 and EMG_1, it may be concluded that this decrease can be outweighed 25 msec later by the facilitatory influence upon ACh release exerted by the arrival of the fourth impulse volley at the junctions although these impulses must have further depleted the transmitter stores This observation strongly suggests that the drop of the EMG_4/EMG_1 ratio to below unity in this phase results from a decrease in the number of free ACh receptors available at 40 msec after the arrival of the third volley of impulses

These differences in amplitude between the electrical responses to the individual pulses of such trains become even more marked in the next period which again, starts after the application of some 200 pulses and is characterized by a gradual slowing down of the

For this reason the experiments described in this section (and in section 3 1 1) were repeated with the E and the L preparations obtained from another 4 frogs, the only difference being that the application of the third pulses of the trains was advanced by 15 msec, so that the pulses now followed each other at intervals of 25, 10, 40 and 25 msec, respectively

The data for the electrical responses of all 8 preparations were averaged, the results are plotted in Fig 11 From a comparison between Figs 10 and 11 it follows that, in the course of stimulation at the rate of 1 per 10 sec, the amplitude of the electrical response to each pulse in the train changes in a similar way for the two types of test train Again, the ratios between EMG_n and EMG_1 exceed unity at the onset of stimulation and the ratio EMG_2/EMG_1 is lower than that of EMG_3/EMG_1 while that of EMG_5/EMG_1 is still higher, but, now, the EMG_4/EMG_1 ratio is lower than any of the others The latter fact, obviously, has to do with the irregular

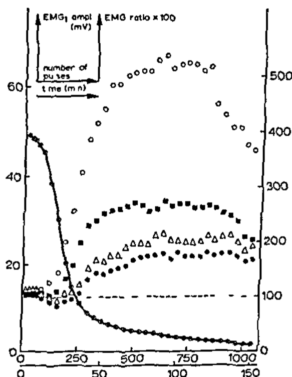


Fig 11 The same as in Fig 10 but now for test trains where the third pulse has been advanced by 15 msec

spacing of the pulses in the trains, which results in a very strong conditioning effect on the responses to the third pulses and a weak one on those due to the fourth pulses After the application of about 100 pulses during which there is a rather slow decrease in amplitude of the responses to single pulses, a period starts in which this amplitude decreases steeply In this period, again, there is a slight lowering of the EMG_2/EMG_1 ratio and even a drop below unity of the EMG_4/EMG_1 and EMG_5/EMG_1 ratios However, the EMG_3/EMG_1 ratio shows a very steep rise suggesting that, at 10 msec after application of the second pulse, the response/stimulus ratio is strongly raised in the units characterized by conditioned facilitation (while it must be markedly lowered in those of the other type) From the behaviour of the EMG_4/EMG_1 ratio it appears that, at 40 msec after application of the third pulses, the response/stimulus ratio of the units with a facilitatory response to conditioning pulses must be lower in the case of the fourth pulses of the trains than in that of the first pulses

If it is assumed that the marked rise of the EMG_3/EMG_1 ratio results from an augmented release of acetylcholine quanta per impulse in the motor units showing conditioned facilitation, then the decrease in amplitude of EMG_4 below that of EMG_1 might be explained by a partial depletion of the stores of readily releasable ACh when the fourth volley of impulses arrives, by a temporary lowering in the number of ACh receptors that are unoccupied at this moment or by both However, from the fact that the EMG_5/EMG_1 ratio is slightly higher than that between EMG_4 and EMG_1, it may be concluded that this decrease can be outweighed 25 msec later by the facilitatory influence upon ACh release exerted by the arrival of the fourth impulse volley at the junctions although these impulses must have further depleted the transmitter stores This observation strongly suggests that the drop of the EMG_4/EMG_1 ratio to below unity in this phase results from a decrease in the number of free ACh receptors available at 40 msec after the arrival of the third volley of impulses

These differences in amplitude between the electrical responses to the individual pulses of such trains become even more marked in the next period which, again starts after the application of some 200 pulses and is characterized by a gradual slowing down of the

rate of decrease of the EMG amplitudes and that at which their ratios increase

After the application of about 700 pulses there is, again a decrease of these ratios This is especially the case for the EMG_2/EMG_1 and EMG_3/EMG_1 ratios, which are raised most strongly by conditioning This suggests that, after 100 min of this *in vitro* stimulation the mechanism where the facilitatory effect originates is deteriorating rapidly

If this should be the case and if the assumption that, in the absence of nervous inflow, an intact blood supply has an after effect preventing deterioration of the mechanism coupling pre synaptic depolarization with ACh release (3 2) is correct, then it is to be expected that the decrease of the EMG amplitude ratios in the experiments would occur earlier in the *E* preparations than in the others

The correctness of this prediction is evidenced in Fig 12 which shows, separately for the *E* and the *L* preparations the changes which occurred in the EMG_2/EMG_1 (left) and the EMG_3/EMG_1 (right) amplitude ratios in the case of the trains in which the third

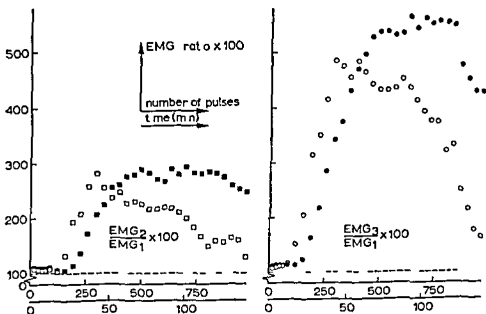


Fig 12 Changes in the amplitude ratios EMG_2/EMG_1 (left) and EMG_3/EMG_1 (right) during indirect stimulation of *E* (open symbols) and *L* (filled symbols) preparations

pulse was advanced by 15 msec From this figure, in which the magnitude of these ratios gives an impression of the effect of conditioning on ACh release, it is evident that the mechanism primarily responsible for facilitation deteriorates, indeed, more rapidly in the *E* preparations than in the others

From the results of a previous investigation it was concluded that the fact that, during indirect stimulation, the mechanical response to a twin pulse is larger than that to a single pulse, is partly due to recruitment of muscle fibres and that the contribution of this factor increases with the number of stimuli applied

Therefore, it is to be expected that the same will apply, *a fortiori*, to the difference between the mechanical responses to single pulses and to pulse trains That this is indeed so, is illustrated in Fig 13 which shows, plotted against the number of pulses applied, the

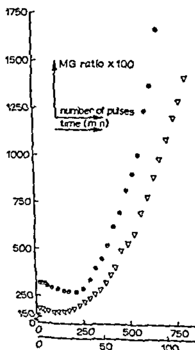


Fig 13 Changes in the ratios between the amplitudes of the mechanical responses to pulse trains (●) or twins (▽) and of those to single pulses during indirect stimulation

changes which occurred in the MG_{1-5}/MG_1 amplitude ratio in the present experiments with single pulses and trains of five pulses at 25 msec intervals, together with those in the MG_{1-2}/MG_1 ratio found earlier (*cf* BOBBERT, *l c*, Fig 3) in experiments with single pulses and 25 msec twins

After the initial decrease of these ratios during the staircase period, both steadily increase From the steeper increase in the case of the MG_{1-5}/MG_1 amplitude ratio it may be concluded that recruitment must indeed play an important role in determining the contraction height during indirect high frequency stimulation of twitch fibres *in vitro*

This strongly suggests that the same applies to the situation *in vivo* with regard to the development of tonic contractions in response to the discharge of motoneurones

From a comparison between the EMG_5/EMG_1 *amplitude ratios* when the pulses are spaced at equal (Fig 10) and at unequal (Fig 11) intervals, it appears that the conditioning effect of the four preceding pulses on the response/stimulus ratio for the last pulse of a train is stronger when they are spaced at equal intervals although in both cases the fifth pulse follows the fourth after 25 msec and the trains are equal in duration and in the number of pulses This gives further support to the conclusion drawn previously that in isolated frog muscles the response of a twitch fibre to a given impulse depends not only on the total number of indirect stimuli applied previously, but also on the temporal pattern according to which the immediately preceding impulses arrived at its motor endplate The consequences of this are illustrated in Fig 14 which pictures for both types of test train the changes in MG_{1-5}/MG_1 amplitude ratio during repetitive indirect stimulation (left) together with the changes in the ratio between the amplitudes of EMG_3 and EMG_1 (right) From this figure it appears that the difference between the ratios of the MG amplitudes of the two types of train corresponds with that between the EMG_3/EMG_1 amplitude ratios while, from a comparison between the curves of the EMG_4/EMG_1 – or the EMG_5/EMG_1 – amplitude ratios (compare Figs 10 and 11) it becomes evident that the difference in MG_{1-5}/MG_1 amplitude ratio between the two types of test train is *inversely* related to the difference in the EMG_4/EMG_1 ratios and, even, to that between those of EMG_5/EMG_1

DISCUSSION

The results of the present investigation give strong support to the conclusion drawn from the previous experiments (BOBBERT, *l c*), that in isolated frog muscles the safety factor for neuromuscular transmission is rather low, instead of high as has repeatedly been asserted (FATT and KATZ, 1951, KRNJEVIĆ and MILEDI, 1958, KATZ, 1962, 1966, BROOKS and THIES, 1962, THESLEFF, 1967)

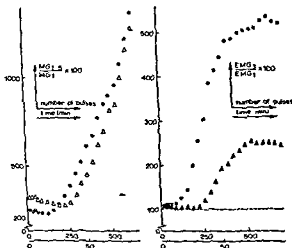


Fig 14 Changes in the ratio between the amplitudes of the mechanical responses to trains of 5 pulses at equal (Δ) and at unequal (●) intervals and of those to single pulses during indirect stimulation, together with the changes in the amplitude ratios EMG_5/EMG_1 (▲ and ●, respectively)

This expresses itself in the fact that, already at the onset of indirect stimulation the response/stimulus ratio is lower than 1 1 for part of the twitch fibres but also in a lowering of this ratio in the course of repetitive low frequency stimulation This decrease has been shown to result both from fatigue and from deterioration due to ageing whose contributions to the change in transfer function were determined previously (BOBBERT *l c*) From the present experiments with E and L-preparations (3 1) it appears that this change in transfer function is retarded if the blood supply to the

muscles has been maintained for a long time after blocking of the physiological nervous inflow. It further appears that this influence, exerted by the circulation after sectioning of the motor nerve, is located at the terminal endings of the *presynaptic elements* and comprises both the presence of larger amounts of immediately available ACh and a higher resistance to deterioration of the mechanism coupling depolarization of the terminal membranes with ACh release (3.2.)

From experiments with direct stimulation it appears that between *E* and *L* preparations there are no appreciable differences in the condition of the electrically excitable part of the twitch fibre membranes, in that of the contractile elements, or in that of the excitation contraction coupling mechanism (3.1.2.). This suggests either that, as long as the circulation is intact, the muscle fibres themselves are hardly fatigued by the physiological nervous inflow, or that their recovery from fatigue is completed within the 15 min elapsing, in the *E* preparations, between blocking of the nervous inflow and interruption of the blood supply.

The presence, in the *L* preparations, of larger stores of readily releasable ACh implies that in intact frogs the normal rate of bloodflow through the calf muscles is inadequate for keeping these stores completely filled and, therefore, that the stores are partially depleted *in vivo*.

Similar observations have been made in cats with regard to the contractile response of the "decentralized" spleen to stimulation of the adrenergic fibres innervating its smooth muscle cells (BROWN *et al.*, 1961, 1966) and on the response of the lateral geniculate nucleus to stimulation of the optic nerves after interruption of the retinal outflow (BURKE and HAYHOW, 1968).

The partial depletion of the ACh stores in the intact frog may result from an insufficient rate of ACh mobilization from mobilization stores (ECCLES, 1957; CURTIS and ECCLES, 1960; BROOKS and THIES, 1962; HUBBARD and WILLIS, 1962; HUBBARD, 1963; HUBBARD and SCHMIDT, 1963; ELMQVIST and QUASTEL, 1965; THESLEFF, 1967) or it may be due to an insufficient rate of ACh synthesis (BIRKS and MACINTOSH, 1957, 1961; BLAIR *et al.*, 1959; STRAUGHAN, 1960; BEANI *et al.*, 1964).

On the other hand, the slower decrease in effectiveness, in the *L*-preparations, of the mechanism effecting the coupling between presynaptic depolarization and transmitter release may be due to an increased aerobic storage of energy which is needed either for the release of calcium ions during presynaptic depolarization, or for binding these ions at their original location afterwards (DEL CASTILLO and KATZ, 1954[a-d], DEL CASTILLO and ENGBAEK, 1954, FELDMAN, 1965, ECCLES *et al*, 1966, KATZ and MILEDI, 1966, 1967, 1968, THESLEFF, 1967, MARUHASHI and WRIGHT, 1967, DODGE and RAHAMIMOFF, 1967, RAHAMIMOFF, 1968, HUBBARD and WILLIS, 1968)

It was concluded from the results of earlier experiments (BOBBERT, *l c*) that with respect to the responses to indirectly applied twin pulses the motor units of the frog's twitch system can be divided into two types, the units of one type show conditioned depression while those of the other type have a facilitatory response While the nature of this difference between the effects of a single condition ing pulse remains unknown it follows from the results of the present experiments (4 5) that units of each type respond in their own peculiar manner to trains of conditioning pulses either with a progressive lowering of the response probability of their twitch fibres or with a continuing rise

Summarizing the results of the present, and of the earlier (BOBBERT, *l c*) experiments it may be stated that the myoneural junctions of the frog s twitch system *in vitro* do not function as simple relays but that the response/stimulus ratio varies widely

It appears that the transfer function of a motor endplate for any given impulse arriving by way of the motor axon depends on

(a) the time elapsed since the axon was sectioned,

(b) the lapse of time since the local circulation was blocked,

(c) the total number of pulses applied after interruption of the blood supply, which determines the degree of junctional fatigue,

(d) the threshold of the axon transmission fatigue develops more rapidly at the junctions of high threshold axons than at those of the (larger) low threshold axons,

(e) the type of motor unit to which the axon belongs, *i e* whether its junctions respond to conditioning impulses with facilitation or with depression This determines whether the

probability of the generation of a postsynaptic spike in response to a given impulse has been increased or lowered by the conditioning action of the immediately preceding impulses,

(f) the number, the overall rate and the temporal pattern of these conditioning impulses,

(g) the interval between the last of these impulses and the impulse in question

Now, it has become probable that during indirect stimulation *in situ*, and perhaps also in intact frogs, the transfer function of the myoneural junctions in the twitch system will be largely determined by the ratio between the magnitude of the local blood flow and the rate of motoneurone discharge *If this should be the case*, it is to be expected that most of the above listed factors will play a rôle in determining the probability of transmission for each presynaptic impulse

SUMMARY

In repetitively stimulated and in ageing frog muscles *in vitro* electrical and mechanical recordings were made from the overall responses to directly or indirectly applied test trains consisting of five equal pulses with varied intervals This was done for *E* preparations in which the circulation had been interrupted shortly after sectioning of the nerves and for *L* preparations in which the circulation *in situ* had been left intact for some 3 h after blocking of the nervous inflow

The rate at which in the course of repetitive indirect stimulation with supramaximal single pulses and pulse trains the electrical and mechanical responses decrease is markedly lower for the *L* preparations than for the *E* preparations (3 1 1)

It appears that this results mainly from a less rapid decrease of the *response/stimulus ratio at the myoneural junctions* of the twitch system in the *L* preparations (3 1 2 3 1 3)

Evidence is adduced that this delayed decrease of neuromuscular transfer in the *L*-preparations *is due to the initial presence of larger stores of readily* releasable acetylcholine and to the later occurrence of deterioration of the mechanism coupling depolarization with release of acetylcholine (3 2)

Already at the onset of stimulation in vitro the response/stimulus ratio for single pulses is lower than 1 1 at part of the myoneural junctions (4)

The rate at which in the course of repetitive indirect stimulation this ratio is lowered in motor units *of the frog s twitch system increases with* the threshold for excitation of their motor axons (4)

Both for the motor units showing conditioned facilitation and for those with depression of the response/stimulus ratio for the second pulses of

twins (BOBBERT, 1969) the conditioning effect of a train of pulses spaced at equal intervals increases with the number of pulses in the train (4, 5)

The changes in the relative amplitudes of the EMG s due to the individual pulses of trains in which the pulses are spaced at equal or unequal intervals, which occur in the course of low frequency indirect activation by supra maximal pulses can be explained from the presence in the preparations of the aforementioned two types of motor units, from a lowering of the response/stimulus ratio in these units caused by repetitive stimulation, and from the fact that the rate at which this ratio decreases is inversely proportional to the excitability of their axons (5)

The results of the present experiments strongly suggest that, in the *intact* frog neuromuscular transmission depends on the relative sufficiency of the blood flow to cope with the pattern of the nervous inflow, and that the responses of twitch fibres to their presynaptic input will vary with both the rate and the pattern of firing of their motoneurones

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SOME CARDIAC EFFECTS OF DIPHENYLHYDANTOIN [1]

BY

E D GERLINGS [2] AND J P GILMORE [3]

INTRODUCTION

Although diphenylhydantoin (Dilantin®) appears to exert its antiarrhythmic effect at dosage levels which apparently have no influence on myocardial contractility, it has been reported that its administration may be associated with myocardial depression (BAUDOUIN and HAZARD 1941, MIERZWAIK *et al*, 1966, MIXTER *et al* 1966) In previous studies it was found that the myocardial depression produced by quinidine was associated with a gain of potassium by the heart (SARNOFF *et al* 1966) This latter observation raised the question as to whether the depression of myocardial performance associated with the administration of Dilantin® was also associated with an alteration of myocardial potassium balance The study described below was undertaken to answer this question The preparation employed also afforded the opportunity to determine the extent to which the changes in myocardial performance induced by Dilantin® are associated with changes in myocardial blood flow and oxygen consumption

MATERIALS AND METHODS

Mongrel dogs of varying weights and of both sexes anesthetized with pentobarbitol sodium (25–30 mg/kg) were used The experimental preparation was an isolated blood perfused dog heart

[1] Supported by Grant HE 10616 01 from the National Institutes of Health U S Public Health Service

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metry and total coronary plasma flow In the more recent experiments the autoanalyzer was put in line so that coronary venous blood could be continuously analyzed for its potassium content All pressures were obtained using Statham transducers and recordings were made on a multichannel direct writing oscillograph Statistical analysis of the data was done using the Student 't test for paired data

In general the experimental procedure was as follows after establishing a hemodynamic steady state simultaneous 30 or 60 sec sampling of coronary arterial and venous blood was started This was continued for 3–5 min at the end of which time the Dilantin® vehicle (40 pCt propylene glycol 10 pCt ethanol and 50 pCt distilled water) or the Dilantin® with vehicle was infused directly into the coronary artery in flow tubing The infusion was maintained for varying periods of time during which samples of coronary artery and venous blood were obtained continuously Timed collections of coronary outflow were made during both the control period and during the infusion of the drug The data presented were obtained from 13 experimental runs in six dogs

RESULTS

The results of these experiments are presented in Table I Experiments 1 and 2 were done using only the Dilantin® vehicle Infusion of the vehicle at an amount equal to 0 97 ml/min into the coronary inflow tubing was associated with essentially no effect on myocardial performance In both experiments however coronary flow increased This increase was quite large in experiment 2 In experiment 2 there was also an augmentation of myocardial oxygen consumption despite a decrease in the coronary A–V O_2 difference The Dilantin® vehicle usually produced an increase in coronary blood flow which if large was occasionally associated with an elevation of myocardial oxygen consumption

Experiments 3–5 (Table 1) demonstrate the effect of Dilantin® plus vehicle on myocardial performance The infusion of Dilantin® was associated with a decrease in both the force (left ventricular developed pressure) and velocity of contraction (dp/dt) Coronary blood flow increased on the average from 40 to 70 ml/min and coronary A–V oxygen difference decreased from 10 4 to 5 4 volumes

similar to that described previously (GILMORE *et al*, 1967) Following the establishment of positive pressure ventilation and transthoracic thoracotomy, the heart was isolated by ligating the inferior and superior venae cavae, azygos vein, lung roots brachiocephalic artery, left subclavian artery and aortic arch immediately below the left subclavian artery The latter was cannulated and connected to either the femoral or carotid arteries of an intact anesthetized dog, thereby providing coronary inflow Total coronary outflow (less Thebesian vein flow) was diverted from the right ventricle by means of a catheter inserted through the azygos vein This catheter in turn diverted the blood through a low resistance rotameter from whence the blood was returned to a reservoir connected to the jugular veins of the support dog Left Thebesian drainage was provided by means of a catheter inserted into the left ventricle through either the apical dimple or left atrium Heart rate was maintained constant throughout any given experiment by stimulation through electrodes sewn either to the right atrium alone or to both right atrium and right ventricle A rubber balloon was tied to the tip of a Y shaped metal cannula which was inserted into the left ventricular cavity That size balloon was used that was judged to approximate the volume of the left ventricular cavity One arm of this cannula served for recording left ventricular pressure while the other arm was used for the addition of fluid The amount of fluid added varied with the size of the balloon Changes in the pressure recorded from the balloon were assumed to reflect changes in left ventricular tension Since balloon volume was maintained constant in any given experiment, myocardial contractility was assumed to have changed if the level and/or rate of change of intraventricular pressure changed Coronary arterio venous oxygen difference was monitored continuously using a Guyton oxygen analyzer and calibration was done by manometric analysis Myocardial oxygen consumption was calculated as the product of the total coronary blood flow and coronary arterio venous oxygen difference

Analysis of coronary artery and venous plasma potassium concentration was done using automated flame photometry Myocardial potassium balance during both the control period and during drug infusion was calculated as the product of the mean coronary arterio venous plasma potassium difference determined by plani

perCent Since coronary blood flow increased less than the decrease in the coronary A–V oxygen difference myocardial oxygen con sumption decreased slightly on the average, from 4 2 to 3 8 ml/min

Since it was apparent that the vehicle itself could influence myocardial oxygen consumption as a result of its influence on coronary blood flow, experiments were done in which the control data were obtained during the vehicle infusion The infusion was then switched rapidly from the vehicle to the vehicle containing Dilantin® while the infusion rate was kept constant (experiments 6–12 Table 1) The infusion of Dilantin® was again associated with a significant decrease in the force of contraction ($P < 0\ 05$) as indicated by the depression of left ventricular developed pressure The maximal velocity of contraction (dp/dt) decreased on the average although no decrease was observed in one of the experiments There was no consistent influence on coronary blood flow However, it will be noted that the control coronary blood flow during infusion of the vehicle was substantially higher than the control blood flows observed in the absence of the vehicle (control experiments 1–5), again indicating the vasodilator effect of the vehicle itself The response of myocardial oxygen consumption was variable On the average it showed no significant change The average A–V oxygen difference for this series also did not change significantly

Experiments 5 and 5a were done in the same animal Experiment 5a was done immediately after experiment 5 but in this instance coronary flow was maintained constant by partial constriction of the coronary inflow tubing In experiment 5 the administration of Dilantin® was associated with a large increase in coronary blood flow and myocardial oxygen consumption However when the same infusion was made in the same heart while keeping coronary blood flow essentially constant (experiment 5a) myocardial oxygen consumption decreased It will also be noted that in experi ment 5 the infusion of Dilantin® had only a modest effect on myocardial performance in terms of both the developed tension and the velocity of contraction however in experiment 5a the administration of the same amount of Dilantin® was associated with a very substantial reduction in both the force and velocity of ventricular contraction (Table 1 and Fig 1)

The influence of Dilantin® on myocardial potassium balance was determined in 7 experimental runs in 5 dogs The response of

TABLE 1[1])

Exp no	H R	LVP mm Hg C[2])	LVP mm Hg I[3])	Max dp/dt mm Hg/sec C	Max dp/dt mm Hg/sec I	CPP mm Hg C	CPP mm Hg I	CBF ml/min C	CBF ml/min I	$A\text{-}V_{O_2}$ Diff vol pCt C	$A\text{-}V_{O_2}$ Diff vol pCt I	MV_{O_2} ml/min C	MV_{O_2} ml/min I	Amount of vehicle (ml/min) or drug (mg/min) infused
							Vehicle							
1	140	148	148	900	800	85	85	85	93	11 0	10 0	9 4	9 3	97 ml/min
2	148	95	95	750	750	80	80	29	57	7 6	5 7	2 2	3 2	97 ml/min
							Dilantin without vehicle control							
3	180	75	35	300	150	50	50	53	55	8 2	5 2	4 3	2 9	3 5
4	140	125	75	750	300	85	60	41	79	14 9	5 6	6 1	4 4	4 9
5	148	110	95	1250	1000	85	75	27	75	8 2	5 5	2 2	4 1	4 9
Mean		103 3	68 3	767	483	73 3	61 7	40 3	69 7	10 4	5 4	4 2	3 8	4 4
S E		12 1	14 4	224	214	9 5	5 9	6 1	6 1	1 8	0 1	0 9	0 4	
5a	148	105	22	900	210	80	—	35	39	8 0	5 8	2 8	2 2	4 9
							Dilantin with vehicle control							
6	168	77	70	1000	1000	68	70	116	100	7 2	9 2	8 3	9 2	5 5
7	168	70	45	1250	500	68	65	128	122	4 2	4 0	5 4	5 6	11 0
8	130	75	67		—	63	65	109	125	2 0	1 0	2 2	1 3	9 0
9	130	75	72		—	62	63	135	141	2 0	1 2	2 7	1 7	9 0
10	110	78	32			50	40	178	131	0 8	0 4	1 4	0 5	19 0
11	156	145	44	1750	750	70	65	150	200	6 5	3 9	9 7	7 8	15 0
12	164	95	23	1250	350	85	80	210	228	3 2	1 2	6 7	2 7	22 0
Mean		87 9	50 4	1313	650	68 8	64 0	140 6	140 6	3 70	3 07	5 2	4 1	12 9
S E		9 2	6 8	136	124	3 7	4 2	12 7	16 3	0 84	1 10	1 1	1 2	
P		$< 0\ 05$		$> 0\ 05$		$> 0\ 1$		$> 0\ 8$		$> 0\ 3$		$> 0\ 1$		

[1]) H R = Heart rate, LVP = left ventricular pressure, dp/dt = first derivative of left ventricular pressure, CPP = coronary perfusion pressure, CBF = coronary blood flow, $A\text{-}V_{O_2}$ Diff — coronary arterio venous oxygen difference, MV_{O_2} = myocardial oxygen consumption

[2]) C = Control [3]) I = During Infusion

It is probable therefore, that the vasodilation observed by others was due not to Dilantin® but to the vehicle

The usual decrease in myocardial oxygen consumption associated with the myocardial depressant effect of Dilantin® was not surprising since it is reasonably well established that when the tension developed by the ventricle is decreased there is an associated decrease in myocardial oxygen consumption What was of interest however, was the occasional observation that despite a decrease in left ventricular developed pressure and coronary blood flow, little change or even an increase in myocardial oxygen consumption was observed The reason for this variability is not known although it is possible that Dilantin® has an oxygen wasting effect The occasional increase in myocardial oxygen consumption observed in the presence of myocardial depression but a substantial increase in coronary blood flow presumably indicates, at least in part, the oxygen supply dependency of the isovolumic blood perfused dog heart In this preparation we observed a direct relationship between coronary blood flow and myocardial oxygen consumption

The failure of Dilantin® to influence myocardial potassium balance even in the presence of quite substantial myocardial depression, differs from the effects that have been found for quinidine (SARNOFF *et al* 1966) These results would indicate therefore that neither the antiarrhythmic nor the depressant effect of Dilantin® is related to changes in myocardial potassium balance and suggests that Dilantin® and quinidine have different mechanisms of action

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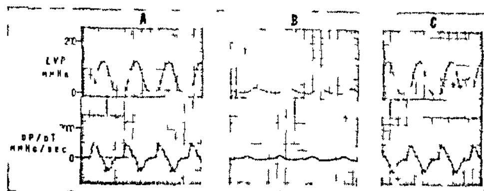


Fig 1

The influence of diphenylhydantoin on some aspects of myocardial performance LVP = left ventricular pressure dp/dt = first derivative of left ventricular pressure A = control tracing B = during intracoronary infusion of diphenylhydantoin (4 9 mg/min), C = following cessation of drug infusion Heart rate and coronary blood flow essentially constant throughout (Table 1, Experiment 13) Paper speed 50 mm/sec See text for further description of figure

DISCUSSION

The observation that Dilantin® can depress myocardial performance is in agreement with that of several other investigators (BAUDOUIN and HAZARD, 1941, MIERZWIAK *et al* 1966 MIXTER *et al*, 1966) The present experiments show conclusively that this depression is a direct effect of the drug itself and not the result of indirect effects elicited through baroreceptor or chemoreceptor mechanisms which might be seen in the intact animal Also the myocardial depression cannot be attributed to the vehicle Several investigators have reported a vasodilator effect of Dilantin® (MIXTER *et al* 1966 and GUPTA *et al*, 1966) However in their experiments no reference is made to the effect of the vehicle alone In the present experiments it was found that the vehicle in which Dilantin® is dissolved is a potent vasodilator but that Dilantin® appears to have no consistent effect on coronary vascular resistance

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SHORT TERM REACTION OF BILIARY BILE ACIDS TO CHOLESTYRAMINE MEDICATION

BY

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1 INTRODUCTION

Cholestyramine is a water insoluble practically unabsorbable strongly basic anion exchange resin which in the intestine binds bile acids thus hindering their reabsorption and promoting their elimination via the feces As a result of this loss of steroid nucleus, cholestyramine can be used to lower serum cholesterol levels in patients suffering from coronary artery disease (BERGEN *et al*, 1959) Our interest in cholestyramine was aroused when we found that in Syrian hamsters it can prevent the formation of cholesterol gallstones and even dissolve such stones once formed (BERGMAN and VAN DER LINDEN 1966 1967)

As it leads to increased fecal loss of bile acids cholestyramine medication gives rise to a condition which as regards bile acids is fully comparable to an external biliary fistula Therefore it has sometimes been called an internal biliary fistula Studies by i a THUREBORN (1962) and DE PALMA *et al* (1967) have shown that in man interruption of the enterohepatic circulation of bile acids by a biliary fistula or other means results in a progressive fall of the bile acid concentration of the bile Still when we studied the effect of cholestyramine on bile composition in man we found no decrease of the total bile acids concentration in samples of bile taken 2 3 days after medication had started nor in those taken 2 weeks later (VAN DER LINDEN and NAKAYAMA 1969) These findings seemed to contradict the results of the studies mentioned above The present study aimed at elucidating this discrepancy by investigating the changes in bile acid concentration during the first 48 hours of cholestyramine medication

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pared with standard curves The *Rf* values of the bile acids were cholic acid 0 17 chenodeoxycholic acid 0 42 and deoxycholic acid 0 47 Samples containing 5 10 15 and 20 μg respectively of cholic acid were chromatographed and so were samples containing similar amounts of deoxycholic - and chenodeoxycholic acids The integrator values obtained for these three bile acids showed a linear relationship up to about 15 μg At higher values the curves leveled off Standards were always run simultaneously and on the same plates as the investigated samples

The precision of the technique was estimated by assaying a pooled bile specimen 16 times on separate days The standard deviations calculated were of a magnitude which showed that although the method is quite usable a certain random error has to be taken into account The mean values obtained were 10 09 mg/ml S D 1 58 for cholic acid 8 19 mg/ml S D 1 11 for chenodeoxycholic acid and 4 76 mg/ml S D 0 45 for deoxycholic acid Further recovery experiments were made by adding weighed amounts of bile acids to a pooled specimen of bile The results showed that with concentrations not surpassing 15 mg/ml the recoveries ranged between 90 pct and 100 pct for cholic acid Those for the other two bile acids were somewhat less ranging between 80 pct and 95 pct for chenodeoxycholic acid and between 83 pct and 93 pct for deoxycholic acid

Comparisons were made in each patient between the initial values and the values found after 24 and 48 hours respectively The significance of these comparisons was tested with the Wilcoxon matched pairs signed ranks test as described by SIEGEL (1956)

3 RESULTS

Fig 1 shows an example of the changes in the biliary bile acid concentration in a patient from the experimental group A more or less similar reaction pattern was found in the other patients of this group During the first day of cholestyramine medication there is a rather rapid fall of the total bile acid concentration Low values persist during several hours and after that there is a gradual rise to values comparable to or somewhat lower than those found before medication started Decreasing values during the 1st day

2. MATERIALS AND METHODS

Sixteen patients, 8 males and 8 females were studied. All had been subjected to surgery for gallstone disease, in the course of which exploration of the common duct had been performed with subsequent insertion of a T drain into the duct. None of them had any biochemical or clinical evidence of liver injury. The investigations were performed while the patients waited for postoperative cholangiography which showed common duct stones to be absent in all patients. The investigations were started at least 4 days after the T tube had been clamped and all outward loss of bile via the T tube or otherwise had ceased. During the investigations the patients were all afebrile, out of bed and fed by mouth.

Eight patients, 4 males and 4 females constituted the experimental group. In this group a sample of 3 ml of bile was taken at 8 a.m. with the patient in the fasting state. Medication with cholestyramine [1]) was then started with a dosage of 4 g four times daily, the last dose being given at 11 p.m. Bile samples of 3 ml were taken every 6th hour during 48 hours.

Under similar conditions and with similar intervals bile samples were taken from the 8 control patients, who were not subjected to cholestyramine medication.

The cholesterol content of the bile was estimated according to a modified Liebermann-Burchard method (HUANG *et al.*, 1961). Phosphorus was determined according to CHEN *et al.* (1956). The phospholipid content was then calculated from the values for lipid phosphorus by multiplying by a factor of 25. Finally, 0.050 ml of bile was extracted and hydrolysed according to the method of WOLLENWEBER *et al.* (1966). According to the same method the free bile acids were separated by thin layer chromatography together with cholic acid, chenodeoxycholic acid and deoxycholic acid standards with the only modification that DC-Fertigplatten Kieselgel F_{254} [2]) were used. After spraying with 15 pct. phosphomolybdic acid in ethanol, the plates were heated to 110 °C for 10 min and the spots were quantified by means of a Vitatron Densitometer U.F.D. [3]) equipped with a 578 $m\mu$ filter and com-

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1) Generously supplied by Merck, Sharp & Dohme, U.S A.

2) Merck, *Darmstadt, Germany.*

3) Vitatron, Dieren, Holland.

change in one patient and a rise in two, the difference between the initial values *and those after* 24 hr of cholestyramine medication did not reach the level of significance During the 2nd day, however, there was a rise in all the patients given cholestyramine and the difference between the values found after 24 and after 48 hr is statistically significant (Wilcoxon $T=0$, $N=8$, $P<0.01$) The deoxycholic acid concentration showed a different pattern

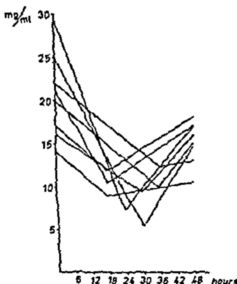


Fig 2
Total biliary bile acid concentrations Initial, lowest and 48 hr values *in* patients treated with cholestyramine

During the 1st day there was a decrease in all patients With the Wilcoxon T test this difference is statistically significant ($P<0.01$) *On the other hand no significant changes were observed during* the 2nd day Alterations, similar to those found for deoxycholic acid, occurred in the chenodeoxycholic acid concentration During *the 1st day* there was a decrease in all patients except in one in which there was a slight rise (Wilcoxon $T=1$, $N=8$, $P<0.02$) No systematic changes were observed during the 2nd day Com-

were observed in all patients Rising concentrations during the 2nd day were found in all patients but one In this patient the decrease had been slow and it took 36 hr before the lowest concentration was reached Later values were somewhat higher but a marked rise was not observed Fig 2 gives the initial values of the total biliary bile acid concentration, the lowest values and the values found after 48 hr The figure shows that the lowest values were observed between 18 and 36 hr after medication had started This lowest value was in 6 cases between 50 and 75 pct of the initial value, in the two others it was considerably lower In the control group no systematic changes in the biliary bile acid concentration were found

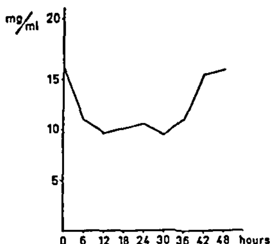


Fig 1
Total biliary bile acid concentration in patient before and during cholestyramine medication

Fig 2 further shows that, although the bile acid concentration rose again during the 2nd day, in 6 of the patients the value after two days of medication was still lower than the initial one In one case similar values were found and in one there was a slight rise When subjected to the Wilcoxon matched pairs signed ranks test, this difference between initial and 48 hr values is probably significant ($T=1$, $N=7$, $0\ 05>0\ 02$)

As seen in Fig 3 the cholic acid concentration decreased in 5 cases, in some of them very substantially. But as there was no

changes The same applied for the cholesterol concentrations of the bile

4 DISCUSSION

As shown by Figs 1 and 2 we found that the typical reaction pattern of the total biliary bile acid concentration during the first 48 hours of cholestyramine medication essentially consists of two parts Its first characteristic is the decrease observed in all patients during the 1st day, although in many of them it was more irregular than in Fig 1 Its second characteristic is the rise which takes place or at least starts during the 2nd day This rise was observed in all but one patient

The decrease in biliary bile acid concentration observed during the 1st and part of the 2nd day of cholestyramine medication – lowest values occurred after 18 to 36 hr – is obviously due to the binding of bile acids by the resin in the intestine It is comparable to that observed in studies in which the enterohepatic circulation of bile acids was interrupted in some other way THUREBORN (1962) for instance found a decrease of the biliary bile acids concentration within 3 hr after he had blocked the common duct by inflating a balloon around the tip of a catheter placed in it In patients with common duct T tubes following gallstone surgery DE PALMA *et al* (1967) found an inverse relationship between the volume of bile obtained and the bile acid concentration When the side arm of the T tube was lowered, loss of bile via this arm increased and the bile acid concentration of the bile fell The opposite occurred when the side arm was elevated i e when the enterohepatic circulation was more or less restored

The rise of the biliary bile acid concentration which starts during the 2nd day was not observed in the studies of interrupted enterohepatic circulation mentioned above In THUREBORN's study the probable reason is that the interruption of the enterohepatic circle did not last long enough for this second phase to occur In DE PALMA *et al* 's study the reason is less clear Both phases the decrease and the subsequent rise of the bile acid concentration were missed in our earlier study (VAN DER LINDEN and NAKAYAMA, 1969) in which, as a rule, the first sample was taken 2–3 days after medication had started In a recent study by SA

parison of the initial and the 48 hr values revealed that the chenodeoxycholic acid concentration had fallen in all patients

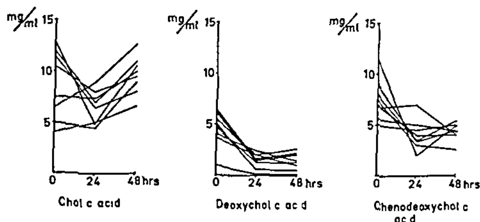


Fig 3

Concentrations of different bile acids Initial 24 hr and 48 hr values in patients treated with cholestyramine

Fig 4 shows the concentrations of the different bile acids found in the control patients who had been treated in exactly the same way as the patients of the experimental group except for the fact that they received no cholestyramine medication No systematic pattern appears in this figure

Neither the phospholipid concentration of the cholestyramine treated group, nor those of the controls showed any systematic

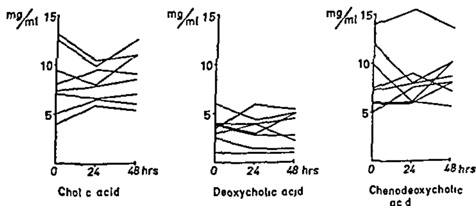


Fig 4

Concentrations of different bile acids Initial 24 hr and 48 hr values in control patients

In the present study we gave 16 g instead of 12 g daily a fourth dose of 4 g being given at 11 p m in order to ensure the resin to be present in the small intestine also during the night As the bile acid values showed an upward trend during the 2nd day one possible explanation for this difference between the two studies is that with a higher dose there is a slower return to normal Our data are however not conclusive on this point and the possibility cannot be excluded that with the higher dose used the compensatory capacity of the liver is nearly exhausted In this connexion it is worthy of note that in hamsters fed a high dose (3 g/100 g food) during 30 days the bile acid concentration calculated as a percentage of total solids was slightly higher in cholestyramine fed animals than in controls (BERGMAN *et al* 1968)

When we turn our attention to the changes found in the ratio between the different bile acids during cholestyramine medication it may be appropriate first briefly to consider the relative merits of the method used for bile acid determination The data we obtained when studying the precision and accuracy of this method show that although it is quite usable its random error is probably somewhat larger than that found by others for more laborious methods According to the statistical consensus there does not however seem to be much need to be overly concerned with this larger error as it will tend to dilute true differences On the other hand caution is needed when a significant difference is not found

As seen in Fig 3 the decrease of the total bile acid concentration in the cholestyramine group affected all three principal bile acids although the level of significance was not reached for cholic acid On the other hand the subsequent rise occurred exclusively in the cholic acid fraction The low concentration of deoxycholic acid is easy to explain Deoxycholic acid is a secondary bile acid formed in the intestine by dehydroxylation of cholic acid Therefore its biliary concentration is bound to fall when reabsorption is hindered More difficult to understand is the observation that the cholic acid concentration rose during the 2nd day whereas that of chenodeoxycholic acid did not One explanation could be that when released from feedback inhibition the liver starts producing predominantly cholic acid The finding of a similar shift towards cholic acid in patients with an external loss of bile (SAMUEL *et al* 1968) is in accordance with this explanation

MUEL *et al* (1968) it was found that, after the insertion of a T tube into the common duct during gallstone surgery the total mass and the concentration of bile acids in the bile decreased during the 2nd and 3rd postoperative days During the 5th day, however, the mass and the concentration increased about fourfold The cause of these phenomena did not become fully clear from their experiments SAMUEL *et al* (1968) *point out that local edema of* tissues, the operative procedure itself of the absence of feeding by mouth postoperatively, may have played a role The same applies to an older study of patients with choledochostomy drainage (EKDAHL and SJOVALL, 1958) These possibilities can be excluded in the present study Controls, treated in exactly the same way, did not show any change in the concentration or percentage composition of biliary bile acids The most probable explanation therefore is a decrease of feedback inhibition of hepatic bile acid neosynthesis which comes into effect some 24 hr after cholestyramine medication has started

ERIKSSON (1957) studied the excretion of bile acids in rats with a bile fistula In these animals there was first a decrease with a minimum occurring after 12 to 18 hr After this minimum a rapid increase was observed These findings in rats with an external loss of bile are comparable to those in our patients who lost bile acids via the gastrointestinal tract The reaction of our patients was, however, more protracted with later minimum values and a slower rise Still, the data suggest that a feedback mechanism of hepatic bile acid production is operative also in man

The question of course arises whether the liver, by an increased *de novo* synthesis of bile acids is able to compensate for their loss, however great this loss may be It appears that the liver can increase its rate of bile salt synthesis only 4 6 fold (HOFFMANN, 1967) A simple calculation based on estimations of bile acid pool size, daily synthesis and number of recirculations shows that this increase will probably not suffice in cases with a complete interruption of the enterohepatic circulation (HEATON 1968)

In the present study comparison of the initial and the 48 hr values revealed a probably significant decrease of the total bile acid values In an earlier study (VAN DER LINDEN and NAKAYAMA, 1969) a similar decresae had not been observed after 48 hr In that study, however, cholestyramine was given in a smaller dose (12 g)

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A difference in affinity could also play a role. In a recent *in vitro* study on the binding of bile salts to cholestyramine JOHNS and BATES (1969) found higher affinity constants with dihydroxy than with trihydroxy derivates. In rats HUFF *et al.* (1963) found that cholestyramine increased the excretion particularly of dihydroxy derivates, they did not differentiate between chenodeoxy- and deoxycholic acid. Of course if also in the intestine cholestyramine has a greater affinity for chenodeoxycholic acid than for cholic acid, the biliary concentration of the former will fall due to its poorer reabsorption. Simultaneously its excretion with the feces will rise. In the present study we found, however, that the concentrations of both cholic acid and chenodeoxycholic acid fell during the 1st day of medication while only that of cholic acid rose during the 2nd. Since affinity cannot change from one day to another, this finding makes it unlikely that differences in affinity and reabsorption could be the only reason for the shift towards cholic acid. Still, it is possible that cholestyramine affects the reabsorption of the two 'primary' bile acids differently in some other indirect and more protracted way, *e.g.* via some influence on the intestinal flora or the rate of fecal evacuation. Experiments aiming at elucidating this problem are in progress in this laboratory.

SUMMARY

Changes in bile composition during the first 48 hr of cholestyramine medication were studied in 8 patients with common duct drains. In all patients all outward loss of bile via the common duct drain or otherwise had finished at least 4 days before the investigation started and none of them had any evidence of liver injury. The typical reaction pattern of the total bile acid concentration was found to consist of essentially two parts. During the 1st day this value decreased rather rapidly. A minimum was reached after 18 to 36 hr followed by a gradual rise. The decrease was most pronounced in the dihydroxy bile acid fraction. On the other hand the rise which took place or at least initiated during the 2nd day was exclusively restricted to the cholic acid fraction.

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AUTHOR INDEX

ARTICLES

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AUTHOR INDEX

ABSTRACTS

Acta Physiol Pharmacol Neerlandica 15
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SUBJECT INDEX

ARTICLES

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SUBJECT INDEX

ABSTRACTS